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ACUTE PANCREATITIS
– STUDIES ON SMOKING AND PROTEASE ACTIVATION

ACUTE PANCREATITIS

Studies on smoking and protease activation

Björn Lindkvist

Akademisk avhandling

Som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligen försvaras i kirurgiska kliniken föreläsningssal Universitets Sjukhuset i Malmö, ingång 42, Torsdagen den 15 december, 2005, kl. 9.15

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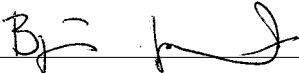
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Title and subtitle ACUTE PANCREATITIS – studies on smoking and protease activation		
Abstract <p>Background and aims: Activation of pancreatic proteases is considered to be a crucial event in the early phase of acute pancreatitis but the cause of this activation is not known. Most cases of acute pancreatitis can be attributed to either gallstone disease or alcohol abuse. However, little is known about other risk factors. The aim of this thesis is to investigate the mechanisms involved in the initiation of acute pancreatitis, trends in the incidence, and risk factors for the disease. The potential role of smoking as a risk factor was given special attention and the effect of nicotine on exocrine pancreas was studied in a rat model.</p> <p>Results and conclusions: Cathepsin B activated trypsinogen but not proelastase or procarboxypeptidase B. Hence, if cathepsin B is to play a role in the activation of digestive enzymes in acute pancreatitis, this probably occurs through activation of trypsinogen. The incidence of gallstone-related acute pancreatitis increased by 7.6% per year (95% confidence interval (CI), 4.0 to 11.4) in Malmö 1985-1999. The incidence of alcohol-related acute pancreatitis decreased by -5.1% per year (95 % CI, -7.4 to -2.8). The risk for acute pancreatitis was increased in smokers (relative risk 2.14, (95% CI, 1.48 to 3.09)), after adjustment for age, sex, body mass index and alcohol consumption. There was a weak association between body mass index and the risk for acute pancreatitis (p=0.02). Nicotine induced increased concentrations of pancreatic proenzymes in pancreatic extract but had no impact on the production of the same enzymes. These findings suggest that nicotine impairs acinar cell secretion. We propose that this might be a contributory mechanism behind the association between smoking and pancreatic disease.</p>		
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Date November 1, 2005

Well in this blues I'm singing,
there's a lesson to be learned.
Don't go around smoking,
unless you want to get burned.

Nick Drake 1967 (*Been Smoking Too Long*)

Tutor	Anders Borgström
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List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

- I **Björn Lindkvist**, Ignacio Fajardo, Gunnar Pejler and Anders Borgström. Cathepsin B activates human trypsinogen 1 but not proelastase 2 or procarboxypeptidase B. *Pancreatology*, in press.*
- II **Björn Lindkvist**, Stefan Appelros, Jonas Manjer and Anders Borgström. Trends in incidence of acute pancreatitis in a Swedish population: is there really an increase? *Clinical Gastroenterology and Hepatology*, 2, 831–837, 2004.**
- III **Björn Lindkvist**, Stefan Appelros, Göran Berglund, Jonas Manjer and Anders Borgström. Smoking is associated with acute pancreatitis—Report from a population-based prospective cohort study. Submitted.
- IV **Björn Lindkvist**, Nils Wierup, Frank Sundler and Anders Borgström. Long term nicotine exposure causes increased concentration of trypsinogens and amylase in pancreatic extracts in the rat. Submitted.

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** Reprinted from *Clinical Gastroenterology and Hepatology*, with permission from the American Gastroenterological Association.

Abbreviations

BAPNA	N α -benzoyl-DL-arg-p-nitroanilide
BMI	body mass index
CAPAP	procarboxypeptidase B activation peptide
CFTR	cystic fibrosis transmembrane conductor regulator
CCK	cholecystokinin
CI	confidence interval
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ERCP	endoscopic retrograde cholangiopancreatography
γ -GT	gamma glutamyle transferase
ICD-8	international statistical classification of diseases, injuries and causes of death (8 th revision)
ICD-9	international classification of diseases (9 th revision)
ICD-10	international statistical classification of diseases and related health problems (10 th revision)
Mm-MAST	Malmö modification of the brief Michigan alcoholism screening test
MPP	Malmö preventive project
mRNA	messenger ribonucleic acid
RIA	radioimmunoassay
RR	relative risk
SIRS	systemic inflammatory response syndrome
SPINK1	serine protease inhibitor Kazal type 1
TAP	trypsinogen activation peptide

Introduction and aims

Acute pancreatitis is an acute inflammation of the pancreatic gland. Case reports describing acute pancreatitis exist from the beginning of the 19th century (1). In 1889, Reginal Fitz published the first structured case series on acute pancreatitis, presenting an accurate description of symptoms, etiology and autopsy findings for 53 patients who died from acute pancreatitis (2). However, no generally accepted definition of the different forms of inflammatory disease of the pancreas was agreed upon before acute, acute relapsing, chronic and chronic relapsing pancreatitis were defined as separate entities at the Symposium of Marseilles in 1963 (3). This definition was later revised with omission of the intermediate relapsing forms for the purpose of clarity (4). Acute onset of abdominal pain in combination with elevated pancreatic enzymes in blood or urine are the diagnostic criteria for acute pancreatitis currently used in clinical practice. Acute pancreatitis can be further defined as mild or severe. The course of the disease is usually benign but 10–25% of all patients will develop the severe form of the disease (5). Mild acute pancreatitis is characterized by a self-limiting course with an uncomplicated and relatively prompt recovery. Severe acute pancreatitis is defined as the development of local complications (e.g. necrosis, abscesses or pseudocysts) or organ failure (6). Severe acute pancreatitis is associated with a high mortality, of approximately 20% (5, 7).

Acute pancreatitis is a common disease. The annual incidence reported in the literature ranges from 7–73/100,000. The two dominating risk factors for acute pancreatitis are gallstone disease and alcohol abuse, which in most studies account for 60–70% of all cases of acute pancreatitis (8). However, in an important proportion of cases, usually 10–30%, no underlying risk factor can be identified (8, 9). This underlines the need for further studies of other potential risk factors for the dis-

ease. Smoking is an established risk factor for pancreatic cancer (10) and chronic pancreatitis (11, 12) but few studies have investigated a potential association between smoking and acute pancreatitis. The biological mechanisms for the association between smoking and pancreatic disease have not been fully explained. Impairment of pancreatic secretion has been demonstrated in smokers (13) and accumulation of pancreatic proenzymes within the pancreas has been shown after nicotine exposure in the rat (14). However studies of the production of pancreatic proenzymes after nicotine exposure are sparse.

Premature activation of the pancreatic proenzyme trypsinogen to the active enzyme trypsin within the pancreatic gland is a crucial event in the initiation of acute pancreatitis. Active trypsin in turn activates pro-inflammatory cascade systems and other digestive proenzymes in the pancreatic juice, leading to inflammation and autodigestion. The cause of this trypsinogen activation is not known. Autoactivation, cathepsin B mediated activation and activation by mast cell tryptase have all been proposed as possible mechanisms.

This thesis investigates factors that influence the initiation of acute pancreatitis. Epidemiological and biochemical methods were used to address this issue in two different ways. The thesis has the following specific aims:

- To investigate the ability of cathepsin B and monomeric tryptase to activate and degrade trypsinogen 1, procarboxypeptidase B and pancreatic proelastase.
- To investigate trends in incidence of acute pancreatitis, and risk factors related to the disease.
- To investigate if smoking or BMI are associated with the risk for acute pancreatitis.
- To investigate the effect of long term nicotine exposure on pancreatic concentration and production of amylase, trypsinogen 1 and trypsinogen 2 in the rat.

Background

Physiology of the exocrine pancreas

The pancreas is located retroperitoneally in the upper abdomen, behind and below the stomach, and is connected to the intestinal tract by the pancreatic duct which opens into the duodenum. From a physiological point of view, the pancreas can be divided into two separate units, one endocrine and one exocrine. The endocrine pancreas regulates energy metabolism by secretion of different hormones into the blood stream and is structurally located in distinct clusters of endocrine cells called the islets of Langerhans, dispersed within the exocrine tissue. The exocrine pancreas constitutes the main part of the gland and its function is to secrete pancreatic juice into the intestinal tract. The pancreatic juice consists of enzymes capable of digesting food nutrients and an isotone fluid rich in bicarbonate that serves to neutralize the acidic gastric secretion.

Secretion of pancreatic juice is under both hormonal and nervous control. The control mechanisms vary during the different phases of digestion. During the cephalic phase, the phase that consists of thinking about, smelling, chewing and swallowing food, the dominant control mechanism is vagal nerve stimulation of the acinar cells (15). This stimulation elicits a moderate enzyme secretion. The next phase is the gastric phase, when ingested food reaches the stomach. This leads to a moderate enzyme output from the pancreas, predominantly mediated by neural stimulation (16). The third and last phase is the intestinal phase which is initiated when food mixed with gastric acid reaches the duodenum. This decreases duodenal pH (17) and leads to release of the hormone secretin (18) which in turn stimulates cells in the pancreatic ducts to secrete water and bicarbonate (19, 20). Another hormone, cholecystokinin (CCK), is released

from the upper small intestine mucosa in response to products of fat and protein digestion (21). The effect of CCK in humans was previously believed to be mediated by binding of CCK to receptors on the acinar cells. However, recent studies have demonstrated that human acinar cells lack CCK-receptors (22). The effect of CCK is instead a stimulation of local afferent nerves that by way of a vago-vagal loop stimulate enzyme secretion from pancreatic acinar cells (23).

The pancreatic juice

Most pancreatic enzymes are secreted as inactive proenzymes (zymogens). Among these are trypsinogen 1 (cationic trypsinogen), trypsinogen 2 (anionic trypsinogen), mesotrypsinogen, chymotrypsinogen, proelastase, procarboxypeptidase A and B, procolipase and prophospholipase A₂. Amylase and lipase are secreted in their active forms. The pancreatic juice is activated when it reaches the duodenum. The activation process is initiated by the cleavage of the trypsinogen activation peptide (TAP) from trypsinogen by the duodenal brush-border enzyme enterokinase. This results in a conformational alteration of the enzyme with exposure of the active site. Trypsin then activates the other pancreatic proenzymes by cleavage of their respective activation peptides (24). Active pancreatic enzymes possess a high proteolytic and lipolytic activity and are capable of causing severe damage if activated within the pancreatic gland. Several protective mechanisms exist to prevent premature activation of the pancreatic proenzymes. Firstly, enzymes are secreted in inactive forms and the proenzymes are not activated until they reach the duodenum. In addition the Serine Protease Inhibitor Kazal type 1 (SPINK1), also referred to as the Pancreatic Secretory Trypsin Inhibitor (PSTI), is secreted along with trypsinogen and is capable of inhibiting trypsin activity if a small amount of trypsinogen is inappropriately activated (25).

Pathophysiology of acute pancreatitis

Clinical and experimental approaches for studies of acute pancreatitis

Research on the pathophysiology of acute pancreatitis suffers from inherent practical problems. Clinically, patients present with a history of abdominal pain lasting from a couple of hours to several days. Clinical studies of the initiation mechanisms of the disease are hampered by the fact that, when patients seek medical care, many of the initiatory pathophysiological events have already occurred. Moreover, the pancreas is not accessible for biopsy or other forms of direct sampling. Acute pancreatitis as a complication of endoscopic retrograde cholangiopancreatography (ERCP) is the only form of human acute pancreatitis that allows monitoring of the whole course of the disease. Studies of patients who develop post-ERCP pancreatitis have provided evidence for early leakage of pancreatic enzymes followed by detection of activation peptides from pancreatic enzymes in urine and increased concentrations of cytokines in blood (26).

Several animal models of acute pancreatitis exist, including induction of increased duodenal pressure and subsequent reflux of bile and pancreatic juice into the pancreas by formation of a closed duodenal loop (27), hyperstimulation with CCK-analogues (28), injection of bile or sodium taurocholate in the pancreatic duct (29, 30) and feeding of mice with choline deficient ethionine supplemented diet (31). A substantial part of our current understanding of the pathophysiology of acute pancreatitis is derived from experiments in animal models like these. However, to what extent these findings can be extrapolated to human acute pancreatitis is still a matter of debate.

From local to systemic inflammation

Today most investigators agree on the importance of trypsinogen activation in the initial phase of acute pancreatitis. The amount of free trypsin activity depends on the amount of trypsinogen activation, inhibitor capacity and trypsin degradation. Factors that might impair this balance during acute pancreatitis and the consequences of free active trypsin are illustrated in figure 1.

In severe acute pancreatitis the local injury leads to a generalized inflammation described as the systemic inflammatory response syndrome (SIRS) similar to what is observed in sepsis, burns, trauma or peritonitis (32). This state is characterized by hypovolemia, hypotension and, in the most severe cases, multiple organ dysfunction and death (32). Hypovolemia is a result of a substantial fluid loss to the retroperitoneal space due to local inflammation as well as to remote organ capillary endothelial leakage and vasodilatation induced by SIRS (33). Hypoxia and hypotension contribute to organ failure and intestinal ischemia, which in turn may lead to endotoxin absorption and possibly also bacterial translocation, although direct evidence from human studies are sparse for the latter (34).

The steps following the initial trypsinogen activation that eventually results in systemic inflammation are not fully understood. The classical understanding of this process emphasizes the role of the pancreatic digestive enzymes. At least in severe acute pancreatitis, the balance between trypsin activity and inhibitor capacity is disturbed to such an extent that free, active trypsin is produced. This leads to activation of different proinflammatory cascade systems and other pancreatic proenzymes. Trypsin has been demonstrated to activate the complement system (35, 36), the kinin system (37, 38), the coagulation and fibrinolytic systems (39) and macrophages (40, 41). However, these are not the sole mediators of the inflammatory process

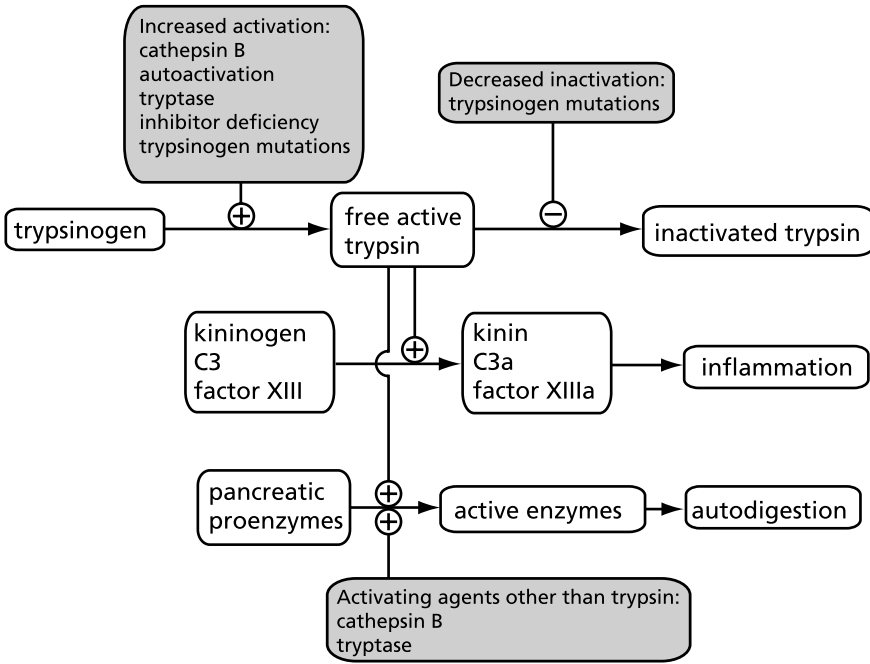


Figure 1. Pathophysiology of acute pancreatitis.

The generation of free active trypsin is a crucial event in the early phase of acute pancreatitis. Active trypsin can in turn activate proinflammatory cascade systems and other pancreatic proenzymes causing inflammation and autodigestion. Mechanisms that might impair the balance between trypsin activity and inhibitor capacity or that might cause increased activation of pancreatic proenzymes by direct activation are presented in shaded boxes.

in acute pancreatitis. A plethora of different pathways and cascade systems have been shown to contribute to the development of systemic inflammation in acute pancreatitis, but the interplay between the different pathways is complex and their relative importance is yet to be settled. Consequently, cytokines, chemokines, reactive oxygen species and lipid mediators are all involved in the progression of the disease, both by their pro and anti inflammatory effect. This subject has been reviewed recently and is beyond the scope of this thesis (42).

Protease activation in acute pancreatitis

In 1896, Chiari introduced the concept of “autodigestion”, proposing that inflammation

and necrosis associated with acute pancreatitis is due to premature activation of pancreatic enzymes within the gland itself (43). Research during the 20th century has provided evidence supporting this hypothesis. The main supporting arguments for the importance of protease activation in acute pancreatitis are that trypsin levels are increased in patients with acute pancreatitis, that markers of trypsin activity correlate with the severity of acute pancreatitis and that activation of trypsinogen within the pancreatic acinar cell has been demonstrated in different models of acute pancreatitis. Trypsin in complex with α_1 -protease inhibitor has been demonstrated in serum of patients with acute pancreatitis and the concentration of the complex is correlated to the severity of the disease (44, 45). The activation peptides of tryp-

sinogen in urine (46, 47), and procarboxypeptidase B in urine and serum (48, 49) have been measured in patients with acute pancreatitis. High concentrations of these peptides are also indicative for a severe course of the disease. The risk for ERCP-induced pancreatitis can be reduced by administration of the serine protease inhibitor gabexate prior to the procedure (50). The severity of experimental pancreatitis can be reduced by prophylactic administration of trypsin inhibitors like aprotinin (51), SPINK1 (52) or gabexate (53). Activation of pancreatic proenzymes has been demonstrated in a variety of experimental models of acute pancreatitis and studies of isolated pancreatic acini have demonstrated that this can occur within the acinar cell (54, 55).

Further studies have examined when, where and how, the protease activation occurs. Today there is a substantial amount of data supporting the theory that activation of trypsinogen and other proteases is a very early event in the course of acute pancreatitis. Elevation of trypsin 2 in complex with α 1-antitrypsin in serum (56) and the activation peptide of procarboxypeptidase B (CAPAP) in urine (26) has been demonstrated within the first 24 hours after the onset of ERCP induced pancreatitis. Elevated concentrations of TAP as early as 15 minutes after induction of pancreatitis have been observed in experimental studies of acute pancreatitis in the rat (57). Treatment with protease inhibitors can ameliorate the course of experimental acute pancreatitis and ERCP-induced pancreatitis but only if administered as a prophylactic treatment (50, 53, 58). Studies of therapeutic administration of protease inhibitors in manifest acute pancreatitis have been notoriously discouraging (59). This also suggests that trypsin activity is most important in the initiation phase of acute pancreatitis. When the patient presents at the clinic, the pathogenic process is probably no longer driven by protease activity but rather by other mechanisms (i.e. inflammation, SIRS) that may or may not have been activated by trypsin earlier on.

Interstitial, intraductal and intracellular activation have all been suggested as potential sites for trypsinogen activation. Leakage of trypsinogen to lymph and blood has been demonstrated in experimental models of oedematous (mild) pancreatitis (60, 61). Intravenous or interstitial addition of enterokinase in these experiments have been proven to produce a fulminant hemorrhagic pancreatitis (61, 62). From these findings, it seems that leakage of trypsinogen and other proteases to the interstitium occurs in mild pancreatitis. The extracellular activation of trypsinogen would then be the turning point that eventually leads to severe acute pancreatitis. Intraductal activation is another theoretically possible mechanism of trypsinogen activation. However, to date there is no strong evidence for this hypothesis. Intraductal activation of trypsinogen by infusion of enterokinase does not induce pancreatitis and elicits only minor pancreatic damage (62). The possibility of intracellular activation of trypsinogen has been explored during the last decades (63, 64). In vivo studies of cerulein-induced acute pancreatitis have demonstrated by subcellular fraction techniques accumulation of TAP in a fraction where heavy granules such as secretory granules and lysosomes normally accumulate (54). In the same model of acute pancreatitis, electron microscopy with immunogold labelled antibodies directed against TAP have demonstrated the appearance of TAP in intracellular vacuoles already 30 minutes after infusion of cerulein. Theoretically, there are several different mechanisms by which trypsinogen could be activated, evidence for these different theories is reviewed below. Cathepsin B mediated activation of trypsinogen requires a pH below 5.0 (65). Since the compartment where such an acidic environment is likely to be found is within intracellular vacuoles appearing in the early phase of acute pancreatitis, the argument for cathepsin B mediated activation can be regarded as an argument for intracellular activation of trypsinogen.

Autoactivation of trypsinogen

The ability of the trypsinogens to autoactivate is well documented in *in vitro* studies performed several decades ago (66–68). Colomb *et al.* demonstrated that purified human trypsinogen autoactivates at pH 8.0 in the presence of calcium. A small but significant trypsin-like activity of trypsinogen was hypothesised to be the initiating factor. This idea was based on the observation of formation of complexes between the “basic trypsin inhibitor” (today referred to as aprotinin) and trypsinogen, indicating the pre-existence of an active site in the trypsinogen molecule (67). Similarly, acetyl-trypsinogen, a trypsinogen variant incapable of activation, was demonstrated to activate chymotrypsinogen at a slow rate, approximately 10^{-5} of expected trypsin activity (68). However, there are some prerequisites for trypsinogen autoactivation. Neutral pH (69) and a calcium concentration of around 1 mM (70, 71) is needed for optimal autoactivation rate of trypsinogen 1. In addition, an inhibitor free environment is required, the presence of SPINK1 decreases the autoactivation rate substantially (72). It is uncertain if these criteria can be met under physiological conditions.

Cathepsin B

Cathepsin B is a cystein protease that is found in most cells within a type of intracellular compartment called the lysosome (73). During normal conditions both lysosomal hydrolases and secretory proenzymes are synthesized on ribosomes attached to the endoplasmic reticulum (74). The peptide chains are then transported through the endoplasmic reticulum to the Golgi apparatus where secretory enzymes are sorted to secretory granules (74) and lysosomal hydrolases to lysosomes by the mannose-6-phosphate pathway (75). Activation of trypsinogen by cathepsin B was first described in studies of bovine enzymes by Green-

baum *et al.* (76) and later on for human enzymes by Figarella *et al.* (77). If cathepsin B is to play a role in the activation of trypsinogen during acute pancreatitis, this intracellular separation of cathepsin B and the digestive enzymes must be disrupted in some way. Steer *et al.* proposed a possible mechanism for this, usually referred to as the “co-localization theory” (63). In many different models of acute pancreatitis, this research group has demonstrated impairment of acinar cell secretion and redistribution of proteases from secretory granules and cathepsin B from lysosomes to intracellular vacuoles that appear in acinar cells during experimental acute pancreatitis (54, 78–80). A low pH is necessary for cathepsin B activity, which has a narrow pH optimum around 4.2 and almost no activity above pH 5.2 (65). Studies of acute pancreatitis in two different animal models (induced by choline-deficient, ethionine-supplemented diet or cerulein) have shown evidence for acidification of these acinar cell vacuoles (81). Additional studies have demonstrated that TAP can be detected in vacuoles during the course of acute pancreatitis (54). Based on the co-localization theory, it was postulated that inhibition of cathepsin B activity would prevent or reduce the severity of experimental acute pancreatitis. It has been demonstrated that addition of a specific cathepsin B inhibitor can prevent trypsinogen activation in studies of supramaximal stimulation of isolated rat acini (82). A specific cell permeable cathepsin B inhibitor L-3-trans-(propylcarbonyl)oxirane-2-carbonyl-L-isoleucyl-L-proline methyl ester (CA-074me) was recently shown to reduce the extent of pancreatic trypsinogen activation and disease severity in two different models of experimental acute pancreatitis *in vivo* (83). However, studies of cathepsin B inhibitors have not been entirely conclusive. Some investigators have not been able to show that cathepsin B inhibition has any beneficial effect on the course of acute pancreatitis (55, 84). A recent study of acute pancreatitis in-

duced by supramaximal cerulein stimulation in cathepsin B deficient knock-out mice has further developed this line of inquiry. Both trypsin activity and pancreatic damage were significantly reduced in the knock-out mice in this study. However, some trypsin activity still occurred and acute pancreatitis was not prevented. Furthermore, inflammation in pancreas and lungs in the knock-out mice was not diminished (85). Theoretically, cathepsin B activation of pancreatic proenzymes other than trypsinogen can occur either indirectly by activation of trypsinogen or by direct activation by cathepsin B. A recent study by Halangk *et al.* investigating the effect of specific trypsin inhibitors in isolated rat acini have provided some indications for a direct activation of pancreatic proenzymes by Cathepsin B (86). Inhibition of trypsin activity led to an increased yield of trypsin after supramaximal CCK-stimulation. The authors propose a protective role for active trypsin by degradation of active proteases, including trypsin itself (86). Consequently, trypsin might not be the only mediator of pancreatic proenzyme activation, in this context, cathepsin B has been proposed. In a previous study, bovine cathepsin B was demonstrated to activate porcine proelastase (87). However, we know of no studies that investigate the action of human cathepsin B on human pancreatic proenzymes other than trypsinogen.

Mast cell tryptase

The importance of mast cells during the early phase of acute pancreatitis has been emphasized in previous studies (61). During the early course of acute pancreatitis there is a basolateral leakage of trypsinogen (61) to the pancreatic interstitium, where the majority of the pancreatic mast cells are found (88). Plasma exudation in the pancreas, colon, and lungs is significantly reduced if the mast cell stabilizer sodium cromoglycate is administered during induction of acute pancreatitis in the rat (89).

Tryptase is a tetrameric serine protease stored in the mast cell secretory granules. It is structurally related to trypsin and shows an overlapping substrate specificity (90). Tryptase has been shown to form tetramers with the active site directed towards the central pore of the tetramer (91, 92). This pore is not accessible to large substrates or inhibitors, e.g. trypsinogen. However, formation of active monomers of human tryptase under physiological conditions has recently been demonstrated. These monomers exhibit markedly enhanced activity towards macromolecular substrates (93). The trypsin-like activity of active tryptase monomers and the location of mast cells in the pancreatic interstitium implies that the active tryptase monomer is theoretically a possible activator of pancreatic proenzymes, including trypsinogen.

Hereditary pancreatitis

Hereditary pancreatitis is a rare condition characterized by recurrent episodes of acute abdominal pain starting in childhood, followed by the development of chronic pancreatitis in the third to fourth decade of life. Subjects with the disease have a dramatically increased risk for pancreatic cancer (94). Hereditary pancreatitis was first described clinically in 1952 (95). In 1996, Whitcomb *et al.* published the first report on genetic mutations associated with the disease (96). In this paper the most common mutation associated with hereditary pancreatitis is described, an Arg-His substitution in the trypsinogen 1 gene at residue 122 (R122H), according to the trypsinogen nomenclature (97). Since then several other mutations in the same gene have been described, including A16V, D22G, K23R, N29I, N29T and R122C (98–100). The pathogenetic mechanisms behind the described mutations have been investigated *in vitro* on recombinant trypsinogen 1. Increased autoactivation and decreased autocatalysis have been demonstrated in the mutated tryp-

sinogens, although the definitive importance of these observations is unclear (65, 71, 72, 100–103). A N34S mutation in the SPINK1 (also known as pancreatic secretory trypsin inhibitor (PSTI)) gene (104, 105) and mutations in the cystic fibrosis transmembrane conductor regulator (CFTR) gene (106, 107) are also associated with hereditary pancreatitis. Whatever the mechanism behind these mutations might be, the characterization of mutations in the trypsinogen 1 and SPINK1 genes speaks for an imbalance between trypsin activity and inhibitor capacity in the pathogenesis of acute pancreatitis.

Epidemiology

Incidence

Annual incidence of acute pancreatitis reported in the literature ranges from 7 to 73 per 100,000 (9,108–117) in publications from the last twenty years. This wide range can, to some extent, be attributed to the inclusion or exclusion of relapses of acute pancreatitis in different studies. Several reports suggest an increased incidence of acute pancreatitis over the last decades (108–110, 112, 116, 118–123). A plotting of all major studies of the incidence of acute pancreatitis from the last thirty years (7, 9, 108–113, 115–121, 124, 125) also indicates an increasing trend over time (figure 2).

Suggested explanations for this trend are changes in the prevalence of established risk factors, i.e. gallstone disease and alcohol abuse (108–110, 116, 119, 122, 126, 127). Improved diagnostic procedures may also have contributed, at least in the earlier decades (119, 123). The definition of the population at risk and retrieval of all cases are crucial tasks when the incidence of a disease is to be investigated. Cases from outside the population at risk might contaminate the data, resulting in overestimation of incidence rates. Alternatively, it is possible that cases within the studied

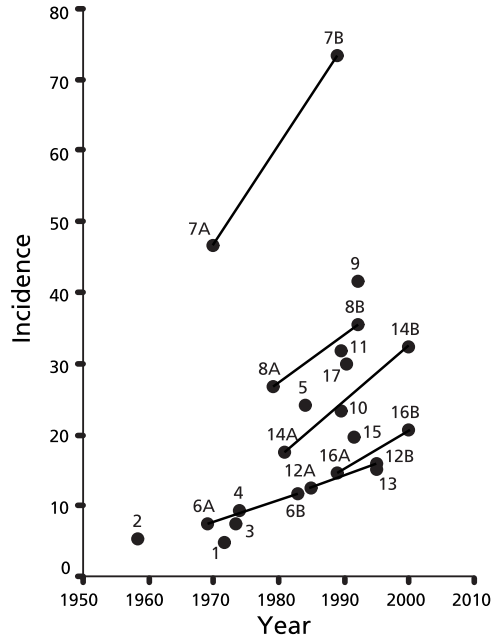


Figure 2. Incidence of acute pancreatitis.

Incidence of acute pancreatitis reported during the last five decades. Points represent centre of the investigated interval, except for when A, signifying the beginning of a period or B, signifying the end of a period, are attached to the given number. 1: Bourke *et al.* 1975 (124), 2: Trapnell *et al.* 1975 (119), 3: Corfield *et al.* 1985 (108), 4: Thomson HJ *et al.* 1985 (111), 5: Thomson SR *et al.* 1987 (125), 6: Giggs *et al.* 1988 (120), 7: Jaakkola *et al.* 1989 (109), 8: Worning *et al.* 1994 (118), 9: Halvorsen *et al.* 1996 (113), 10: Appelros *et al.* 1999 (9), 11: McKay *et al.* 1999 (112), 12: Eland *et al.* 2000 (110), 13: Toh *et al.* 2000 (117), 14: Floyd *et al.* 2002 (121), 15: Lankisch *et al.* 2002 (115), 16: Tinto *et al.* 2002 (116), 17: Andersson *et al.* 2004 (7).

population might be lost because of incomplete registers or if treated in another hospital. Both these situations would lead to underestimation of incidence. Malmö is a city in the south of Sweden with a population of about 250,000 inhabitants. There is only one hospital in the city and there are no referrals of patients to or from this hospital, providing good

conditions for complete case retrieval. Exact population statistics are available.

Risk factors for acute pancreatitis

The two major established risk factors for acute pancreatitis are gallstone disease and alcohol consumption. Together they account for about 70% of all cases of acute pancreatitis in most reports (122). In an important proportion of cases, no underlying risk factor can be identified, this group usually accounts for 10–30% of all cases of acute pancreatitis (128). Leaving out acute pancreatitis of unknown etiology, ERCP-induced acute pancreatitis is the third most common etiological group of acute pancreatitis (9). Several other conditions have been reported to increase the risk for acute pancreatitis but they only contribute to a minor fraction of the total number of cases. Among these are postoperative acute pancreatitis, acute pancreatitis associated with hyperlipidemia, hypercalcemia, pancreatic duct anomalies, viral infections and toxic consequences of different substances including drugs (8, 9). Previous reports have suggested that overweight may be a prognostic factor in acute pancreatitis (129–132), but it has not been proven to be a risk factor for the disease.

Smoking and acute pancreatitis

Smoking is associated with pancreatic cancer and chronic pancreatitis

Pancreatic cancer is the form of pancreatic disease that is most consistently associated with smoking. Most studies have reported approximately a doubled relative risk (RR) for developing pancreatic cancer for smokers as compared to never smokers (133). A dose-response relationship between smoking and pancreatic cancer has also been established (10). An as-

sociation between smoking and chronic pancreatitis has been described in several studies (11, 12, 134, 135). Smoking has also been suggested to accelerate the progression of chronic alcohol-related pancreatitis. In a retrospective study of a cohort of patients with alcohol-related chronic pancreatitis, smoking was shown to be associated with an increased risk for pancreatic calcifications and diabetes as well as with younger age at diagnosis (136).

Association between smoking and acute pancreatitis

Confounding by alcohol consumption is an inherent problem in all epidemiological studies of a possible association between smoking and acute pancreatitis. This is due to the fact that alcohol consumption is known to be higher among smokers than non smokers (137, 138), and that alcohol is an important risk factor for acute pancreatitis (9). Two case-control studies have found evidence for an association between smoking and acute pancreatitis. Talamini *et al.* described an association between smoking and acute pancreatitis in a study where smoking habits among patients with alcohol-related acute pancreatitis were compared with smoking habits among control individuals who were randomly selected from the background population (134). Blomgren *et al.* found that smoking was significantly associated with acute pancreatitis in a study in which patients hospitalized with an increased amylase level were compared to controls randomly chosen from a population register (139). The selection of control subjects is a major problem connected to the case-control design and it may be difficult to extrapolate findings from such studies to the general population. Cohort studies provide more reliable, representative controls and the validity of risk factor assessment is considered high compared to other types of studies. To date, there is only one cohort study that investigates the possible association between smoking and pancreatitis, re-

cently published by Morton *et al.* (140). In this study smokers of more than one pack of cigarettes a day were found to have a relative risk (RR) of 4.9, (95% confidence interval (CI) 2.2 to 11.2) for alcohol-related pancreatitis and a RR of 3.1 (1.4 to 7.2) for idiopathic pancreatitis. There was no statistically significantly increased risk among smokers for gallstone-related pancreatitis (RR 1.3, 0.5 to 3.1). In this study, subjects with signs of chronic pancreatitis at first hospitalization were not excluded from the analysis. Subgroup analysis revealed that the relationship between smoking and the risk for pancreatitis was present also when patients with signs of chronic disease were excluded, however, this latter analysis was not controlled for alcohol consumption.

Acute pancreatitis is not an established side effect of pure nicotine exposure. There is one case-report describing a case of acute edematous pancreatitis after nicotine intoxication (estimated to 38 mg) by the combination of cigarette smoking and a nicotine patch in a patient where no other risk factors were identified (141). In another study of the possible therapeutic effect of high dose (up to 22 mg) nicotine treatment in patients with ulcerative colitis, one case of acute pancreatitis was described after treatment onset (142).

Effect of smoking on exocrine pancreas

The effect of smoking and nicotine on pancreatic secretion and levels of pancreatic enzymes in blood have been studied in man. Smokers have a decreased output of pancreatic juice during stimulation by secretin (143). The smoking of one single cigarette has been shown to elicit an immediate decrease in the output of pancreatic fluids and bicarbonate (144). Leakage of pancreatic proenzymes into the bloodstream has been demonstrated both before and after secretin administration in smokers (13). The effect of nicotine has been further studied in animal models; most inves-

tigators have used the rat with different administration routes of nicotine. Some of the more important studies and their findings are summarized in table 1 (14, 145–154).

There is agreement about nicotine's morphological effect on the pancreas. Vacuolization and edematous swelling of acinar cells have been reported after three different modes of nicotine administration (oral, subcutaneous and aerosol) (14, 148, 149, 152). From the functional point of view, results are somewhat divergent. Most investigators have demonstrated increased levels of pancreatic enzymes in pancreatic extracts after nicotine exposure (14, 145, 152). However, secretagogue stimulated release of pancreatic enzymes has been shown to be both increased (145, 153) and decreased (14, 146, 151, 152). It has been proposed that nicotine in itself can induce amylase release from acinar cells (153), but these findings were not reproducible when pH in the medium was kept constant (151). There are few studies that have addressed the question of the nicotine effect on the synthesis of pancreatic enzymes. These studies have provided some indications for an increased protein synthesis by the demonstration of increased amylase mRNA levels (145, 152) and increased incorporation of radiolabelled leucine (153).

In summary, there is substantial evidence for an impairment of exocrine pancreatic function by smoking and nicotine. The accumulation of pancreatic proenzymes and the disturbed response to secretagogues provide a possible pathophysiological explanation for an association between smoking and pancreatic disease.

Smoking, alcohol consumption and overweight in the population at risk

In Sweden, there has been a general decrease in smoking, especially among men, during the last 25 years (figure 3) (155). Smoking among women is also decreasing but this is due to a decrease in the younger age groups. There is

Table 1. Experimental studies of the effect of nicotine exposure on exocrine pancreas in the rat.

Reference	Author	Nicotine administration	Reported effect of nicotine
150	Hartwig <i>et al.</i>	In vivo, acute administration of cigarette smoke in combination with alcohol to anaesthetized rats.	<ol style="list-style-type: none"> 1. Decreased pancreatic blood flow. 2. Increased leukocyte-endothelium interaction. 3. Increased leukocyte sequestration in pancreatic tissue. 4. Potentiation of alcohol induced impairment of pancreatic blood flow.
14	Chowdhury <i>et al.</i>	In vivo by drinking water (0.77 mM) for 28 days.	<ol style="list-style-type: none"> 1. Cytoplasmic vacuolization and cellular edema. 2. Decreased CCK8 stimulated amylase release. 3. Increased total cellular amylase content. 4. No effect on membrane binding capacity or dissociation constant for the CCK-receptor. 5. Decreased weight gain. 6. Decreased plasma glucose and insulin.
154	Doi <i>et al.</i>	In vitro (0–1 nM) on isolated pancreatic acini.	<ol style="list-style-type: none"> 1. Nicotine does not bind to surface receptors on acinar cells. 2. Nicotine accumulates intracellularly. 3. Carbachol and CCK significantly enhanced intracellular accumulation of nicotine.
148	Chowdhury <i>et al.</i>	In vivo, by nicotine aerosol for 21 days.	<ol style="list-style-type: none"> 1. Vacuolization of acinar cells. 2. No changes in glucose, gastrin, and CCK.
146	Chowdhury <i>et al.</i>	In vivo by gavage tube (0.12 mmol/kg*day) for 120 days.	<ol style="list-style-type: none"> 1. Food intake unaffected. 2. Decreased body weight. 3. Increased postprandial plasma gastrin. 4. Decreased food-stimulated output of trypsin.
149	Chowdhury <i>et al.</i>	In vivo by drinking water (50 mg/L and 200 mg/L) for 16 weeks and by drinking water (200 mg/L) for 12 weeks followed by 4 weeks of normal water.	<ol style="list-style-type: none"> 1. Dose dependent decrease in body weight gain and food and fluid intake. 2. Increased plasma levels of CCK. 3. Decreased secretory response to CCK. 4. Vacuolization of pancreatic cells. 5. Complete reversal of metabolic parameters and partial recovery of histological changes after nicotine withdrawal.

Table 1. *Continued*

Reference	Author	Nicotine administration	Reported effect of nicotine
152	Lau <i>et al.</i>	In vivo by time-release pellets (5–50 mg) for 3–12 weeks. Plasma nicotine 76 ng/ml, cotinine > 300 ng/ml.	Treatment with 50 mg for 12 weeks: <ol style="list-style-type: none"> 1. Increased trypsin and chymotrypsin activity in pancreatic extracts. 2. Increased amylase mRNA levels in pancreatic extracts. 3. Decreased CCK8 secretory response. 4. Vacuolization of acinar cells (appearing after 3 weeks). 5. No effects on body weight.
147	Chowdhury <i>et al.</i>	In vivo administration of nicotine by drinking water for 16 weeks. In vitro (10 μ M–30 mM) on isolated acinar cells.	<ol style="list-style-type: none"> 1. Inhibition of carbachol-stimulated amylase release.
151	Hosotani <i>et al.</i>	In vitro (10 μ M–30 mM) on isolated pancreatic acini.	<ol style="list-style-type: none"> 1. No effect on basal non stimulated amylase release when keeping pH constant. 2. Inhibition of carbachol-stimulated amylase release.
145	Dubick <i>et al.</i>	In vivo by time-release pellets (1.65 μ g/min) for 3 weeks.	<ol style="list-style-type: none"> 1. No effects on body weight. 2. Increased activity of amylase, trypsin and chymotrypsin in pancreatic extracts. 3. Increased immunoreactive cationic trypsin in pancreatic extracts and serum. 4. Increased amylase mRNA in pancreatic extracts. 5. Increased basal and secretagogue induced secretion of amylase, trypsin and chymotrypsin.
153	Majumdar <i>et al.</i>	In vitro (3–25 mM) on isolated pancreatic acini.	<ol style="list-style-type: none"> 1. Increased release of trypsinogen and amylase. 2. Increased production of pancreatic enzymes (shown by incubation with radiolabelled leucine).

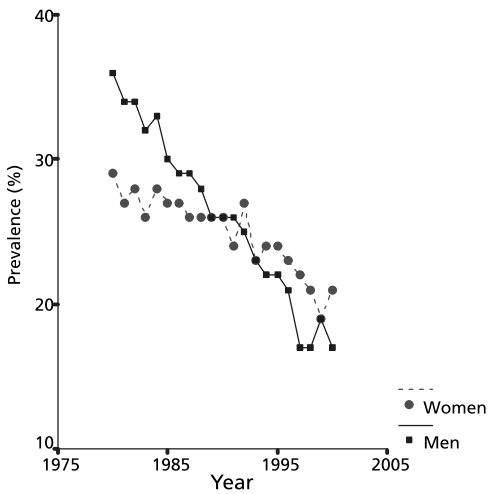


Figure 3. Prevalence of smoking among men and women above 15 years of age in Sweden.

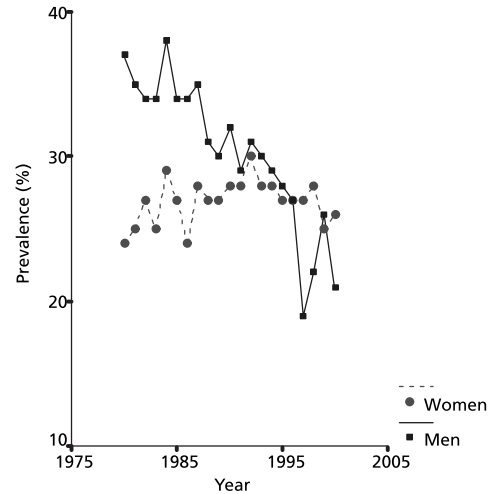


Figure 4. Prevalence of smoking among men and women, 45–64 years of age in Sweden.

no significant decrease in the proportion of smokers above the age of 45 years (155).

In the age group with the highest prevalence of acute pancreatitis (45–64 years), there was a marked decrease in the proportion of smokers among men, but no significant trend among women (figure 4) (155). The use of smokeless tobacco is increasing on the national level. Sales of Swedish snuff have increased by almost 50% from 1980 to 2000 according to statistics by the manufacturer Swedish Match (156). There is no specific data on tobacco consumption for the Malmö area.

The total alcohol consumption in Sweden (registered and unregistered including estimates of illegal import) has probably been stable on a per capita basis from 1989 to 1999 according to surveys by Swedish authorities (156). Taxes on alcoholic beverages are high in Sweden and there is a substantial influx of spirits, wine and beer from neighboring countries. The extent of import, both illegal and legal, have most probably not been constant since import restrictions have varied markedly during the last decades. This problem hampers

reporting of statistics on both alcohol and tobacco consumption in Sweden. The impact of this problem may be even greater in Malmö due to the city's geographical proximity to Denmark and continental Europe.

Repeated studies by postal questionnaires have provided some indications for an increasing prevalence of overweight and obesity in the Malmö area during the investigated period. The prevalence of overweight (BMI 25–29.9 kg/m²) increased from 26% to 39% and the prevalence of obesity (BMI >30 kg/m²) increased from 5% to 9% in these surveys (157).

Material and methods

Laboratory methods

Purification of pancreatic enzymes

Pancreatic juice was harvested from patients who had their pancreatic duct drained after pancreatic surgery. The juice was collected for 12 hours at room temperature and then stored

in a -20°C freezer to avoid activation of pancreatic proenzymes. Trypsinogen 1, procarboxypeptidase B and proelastase were all purified from the pancreatic juice using Mono Q, Mono S and affinity columns in different sequences. The procedure for purification of procarboxypeptidase B has been described in detail previously (48). Protocols for purification of trypsinogen 1 and proelastase are presented in paper I.

Methods for measurement of proenzymes and their activation

Several different tools are available for measurement of the concentration and activation of different proenzymes, each method having its own advantages and drawbacks. Active enzymes can be measured by their activity towards fluorogenic substrates, more or less specific for the investigated enzymes. This method was used in paper I and IV, using the substrate $\text{N}\alpha$ -benzoyl-DL-Arg-p-nitroanilide (BAPNA) for trypsin activity, and in paper I using N-succinyl-Ala-Ala-Pro-Leu P-nitroanilide for elastase activity. The advantage of this approach is that only viable enzymes are measured. However, inhibitors and the activity of other contaminating enzymes are often a problem resulting in both over and under estimation of actual protein concentration. Therefore, this way of measuring enzyme concentrations is more reliable in systems of purified enzymes where the concentration of contaminating enzymes and inhibitors is low.

Estimates of proenzyme concentration can either be done by direct methods focused on the proenzyme or indirectly by activation of the proenzyme followed by subsequent measurement of activation peptides or enzyme activity. Activation peptides from pancreatic proenzymes are cleaved from the proenzyme when they are activated. They can be regarded as markers of trypsin activity and they will appear in concentrations equimolar to the concentration of the active enzyme. Proenzymes

and activation peptides can be detected by immunological methods such as radioimmunoassay (RIA) or Enzyme-Linked Immunosorbent Assay (ELISA). These methods have a higher sensitivity compared to methods using enzyme activity. The interpretation of such results can be impaired if proenzymes or activation peptides are degraded in the sample or if degradation of the entire proenzyme exposes the immunoreactive site of the activation peptide. Antibodies are not always entirely specific and inappropriate precipitation of other antigens may also be a problem. A RIA for the human trypsinogen activation peptide (TAP) was used in paper I and RIAs for the activation peptides of rat trypsinogen 1 (TAP 1) and rat trypsinogen 2 (TAP 2) were used in paper IV. ELISAs for procarboxypeptidase B and its activation peptide (CAPAP) were used in paper I. All protocols are described in detail in the corresponding papers.

Radioimmunoassays for the activation peptides of trypsinogen 1 and 2

In paper IV we wanted to measure and compare concentrations of both trypsinogen 1 and 2 in the rat. Since no practical method for this purpose was available, we set out to develop two different RIAs for quantification of the activation peptides corresponding to the two trypsinogens. In the rat, the activation peptide of trypsinogen 1 (TAP 1) differ from the activation peptide of trypsinogen 2 (TAP 2) in two amino acids (TAP 1 = LPLDDDDK and TAP 2 = FPLEDDDK) and production of antibodies specific for each peptide is therefore theoretically possible. Antibodies against TAP 1 and TAP 2 were obtained by immunization of rabbits with synthetically produced, cystein-conjugated TAP 1 and TAP 2, conjugated to keyhole limpet hemocyanin. Iodinated TAP 1 and TAP 2 were used as tracers in the RIAs. Free and bound radioactivity were separated by means of a second step antibody precipita-

tion and radioactivity in the supernatant was counted in a γ -spectrometer. The specificity of the two antibodies was investigated by addition of TAP 1 in the TAP 2 RIA and vice versa and by preparative electrophoresis.

In situ hybridization

In paper IV, mRNA for trypsinogen 1, trypsinogen 2, procolipase and CCK-A receptor were measured by in situ hybridization. This was done in order to provide indirect measurements of how the production of these proteins was affected by nicotine exposure. The analysis was performed according to previously published protocols (158). The sequence specificity of the probes was determined using the GenBank database. No significant similarity with other mammalian mRNAs was found.

Long term nicotine exposure in the rat

Nicotine was administered to rats in the treatment group by addition of nicotine to the drinking water at a concentration of 0.77 mM, as previously described by Chowdhury *et al.* (14). Both groups had food and fluid available ad libitum. After 28 days the animals were fasted over night and anesthetized with a combination of fentanyl fluanisone and midazolam. The pancreas was removed and animals were sacrificed by ex-sanguination. Blood was centrifuged and plasma was frozen. Pancreatic tissue was immediately frozen on liquid nitrogen to avoid activation of pancreatic enzymes. Two rats were excluded from the treatment group because of failure in the DNA analysis and one rat was excluded from the control group because of inadequate sampling of pancreatic tissue. Acidic pancreatic extracts were prepared. Amylase activity was measured in the untreated extracts. Trypsinogen concentrations were measured by first incubating the extracts with enterokinase at neutral pH. TAP 1 and 2 were then measured by the described RIA methods.

Population at risk and cases of acute pancreatitis

Malmö Acute Pancreatitis Data Base

A data-base of all cases of acute pancreatitis in Malmö from 1985 to 1999 was created in order to study trends in the incidence of acute pancreatitis (paper I). The database was also used to provide information on endpoints in a study of risk factors related to the disease (paper III). Case retrieval was performed by computer-aided searches of local clinical and autopsy records and regional forensic records for diagnostic codes for acute pancreatitis (ICD-8: 577,XX, ICD-9: 577A, ICD-10: K859). Forensic and autopsy records and clinical notes were evaluated according to a standard protocol. The diagnosis was accepted in cases with a history of acute abdominal pain in combination with an increased serum amylase or when findings from laparotomy or autopsy were indicative for acute pancreatitis. Cases with a history of chronic pancreatitis or previous attacks of acute pancreatitis were excluded. When present, the judgment of the treating clinician was considered when plausible etiology (gallstone disease, alcohol abuse or other/unknown risk factors) related to the attack of acute pancreatitis was assessed. However, gallstone disease was only accepted as etiology in cases where the diagnosis of gallstones was confirmed by imaging procedures. Similarly, a history of a high regular intake of alcohol or an alcoholic bout prior to the onset of the disease was required for classifying the attack as alcohol-related.

Gallstone disease, alcohol abuse and lung cancer in the population at risk

Trends in the incidence of gallstone disease and alcohol abuse are likely to influence the incidence of acute pancreatitis since these two

conditions constitute the two most important risk factors for the disease (8, 9). In order to obtain estimates of the incidence of these conditions in the background population diagnostic records were sought for relevant diagnostic codes. Only the main diagnosis in each hospital stay was accepted, there was no case validation performed. Cholelithiasis, cholecystolithiasis, and cholecystitis (ICD-8: 574,X, ICD-9: 574X, ICD-10: K80X) were used as markers for gallstone disease. Delirium tremens (ICD-8: 291,00, ICD-9: 291A, ICD-10: F10.4) and mortality from cirrhosis (ICD-8: 571,00, ICD-9: 571C, ICD-10: K70.3) were used as markers for alcohol abuse. In addition to the established risk factors, trends in smoking habits were investigated using the incidence of lung cancer (ICD-8: 162,XX, ICD-9: 162X, ICD-10: C34X) as an indicator in order to reveal a possible relationship between smoking and acute pancreatitis.

Malmö Preventive Project

Malmö Preventive Project was a project set up in 1974 with the main purpose to screen the middle-aged population for cardiovascular

disease risk factors (159). Entire birth-year cohorts were invited between 1974 and 1992 to a health screening investigation, consisting of a physical examination, blood sampling and a self-administered questionnaire of about 200 questions on lifestyle, medical history and heredity. All men born in 1921, in 1926–1942, 1944, 1946 and in 1948–49, and all women born in 1926, in 1928, 1930–1938, 1941 and in 1949, were included. The attendance rate was high (71%) and a total of 33,346 individuals (22,444 men and 10,902 women) were examined within the project (160).

Cases of acute pancreatitis in Malmö Preventive Project

Record linkage of the Malmö Acute Pancreatitis Data Base to Malmö Preventive Project revealed 262 cases of acute pancreatitis in the cohort. Out of these, 179 were found to be incident cases (i.e. subjects without any history of acute pancreatitis at baseline investigation) (figure 5). Subjects with prevalent acute pancreatitis were excluded from the analysis. The aim of study III was to compare this group of cases with acute pancreatitis to the rest of the Malmö Preventive Project cohort in or-

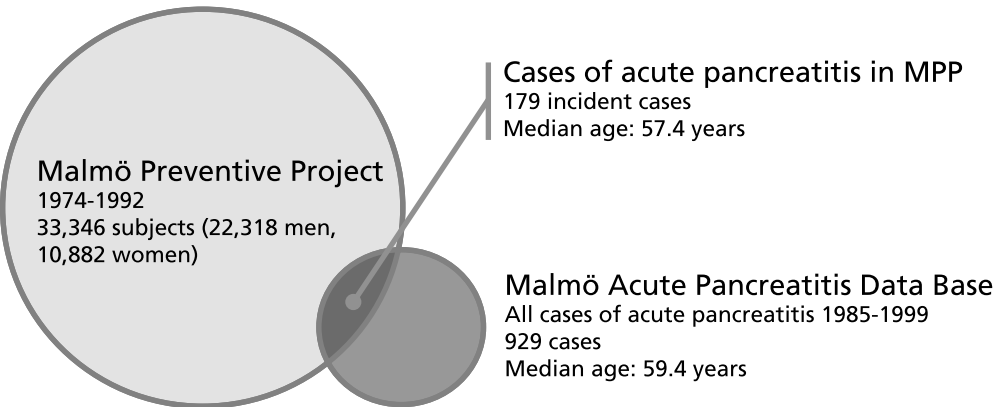


Figure 5. Distribution of cases of acute pancreatitis in Malmö Preventive Project and Malmö Acute Pancreatitis Data Base.

der to investigate risk factors for the disease. Data collected at the base-line investigation was used to assess exposure to potential risk factors in cases and healthy subjects. The following risk factors were evaluated: age, sex, alcohol consumption, BMI and smoking. Data on age, sex and BMI were readily available for virtually all subjects in the cohort. Smoking status was defined using data from the questionnaire. First, subjects were classified as never or ever smokers. Following this, ever smokers were further classified as current or former smokers and smoking dose was assessed by additional questions to current smokers. Alcohol consumption was estimated by two separate means, serum γ -glutamyl transferase (γ -GT) values and a scoring system based on questions taken from a modified version of the Michigan Alcoholism Screening Test (161), referred to as the “Malmö modification of the brief MAST” (Mm-MAST) (162). Subjects were classified as low, intermediate or high alcohol consumers based on their Mm-MAST score. The γ -GT variable was used to divide the cohort into quartiles.

Epidemiological methods and statistical analysis

The methodology in two of the papers in this thesis (paper II and III) relies to a great extent on epidemiological methods and statistical analysis. A summary of applied methods and terms is presented below.

Time trends in incidence

Trends in the incidence of acute pancreatitis related to different associated risk factors (gallstone disease, alcohol abuse and other/unknown) were investigated in paper II. First a correlation analysis was performed. The aim of this procedure was to investigate if there was a trend in the incidence of acute pancreatitis over time. This can be illustrated in a diagram with incidence on the y-axis and time on the

x-axis. In this diagram, the degree of correlation corresponds to how well the yearly incidence is arranged on a straight line. Pearson's correlation coefficient (r) was used to express goodness of fit. The interpretation of Pearson's constant is that $r = 1$ is a perfect correlation and $r = 0$ means no correlation at all between the variables.

Second, the strength of the association was investigated by linear regression. The output of a linear regression is an intercept and a β -coefficient which corresponds to the equation of the straight line ($y = a(\text{intercept}) + \beta x$). Again referring to the diagram with incidence of acute pancreatitis on the y-axis and time on the x-axis, the linear regression reveals the slope of the line (the β -value) which tells us about the extent of the change in incidence per year. In order to facilitate the interpretation of the β -coefficient, logarithmic values of annual incidence were entered in the linear regression analysis. In this calculation e^{β} gives the change in annual incidence rate in percent (163).

The incidence of almost all diseases varies in different age groups. Changes in the age distribution of the investigated population over time could therefore bias the results when time trends in the incidence of a disease are to be studied. This problem was addressed by replacing crude incidence rates by age standardized incidence. Age standardized incidence was obtained by direct standardization using the total population in Malmö in 1985 as standard (164).

Potential risk factors for acute pancreatitis

In paper III the association between different risk factors and the risk for acute pancreatitis was studied. A Cox proportional hazards analysis was used for this purpose. The Cox analysis is a form of survival analysis which takes in consideration entered covariates (exposures), the number of years a certain subject has been under risk for an event and the out-

come. In this case the outcome was whether a subject got acute pancreatitis or not. The Cox analysis produces a relative risk, sometimes referred to as a hazard ratio, with a confidence interval. The relative risk is interpreted as the risk for an exposed individual of getting the disease as compared to an unexposed. Advantages with this model are that it uses all information in the material and adjusts for multiple covariates.

An effort is made in paper III to investigate if smoking is an independent risk factor for acute pancreatitis, or if the observed association between smoking and acute pancreatitis is only the result of the fact that smokers drink more alcohol than non smokers. Such a spurious relationship is usually referred to as confounding. Several different measures were taken in the analysis of the data to investigate this issue. First, in the Cox analysis, adjustment was made for alcohol consumption (as well as for age, sex and BMI) when smoking was studied. Adjustment allows separation of the effect of multiple covariates. The resulting relative risk can be regarded as the risk for an exposed compared to an unexposed individual to get the disease, keeping all other variables constant. Second, the effect of smoking was investigated in groups of individuals who were believed to be homogenous regarding alcohol consumption (e.g. low, intermediate or high consumers according to the Mm-MAST test). This process is referred to as stratification. If there is no confounding between the variables, smoking can be expected to increase the relative risk for acute pancreatitis in every group (strata) of alcohol consumption. Third, interaction between smoking and alcohol consumption was investigated. Interaction between two variables is considered to occur when the combined effect of two variables is different from what could be expected from their individual effects. The interaction analysis between smoking and alcohol consumption was accomplished by entering a so-called interaction term for these exposures in the Cox

analysis. If the interaction term is statistically significantly associated with the risk for acute pancreatitis, when the smoking and alcohol consumption variables are adjusted for in the analysis, there is considered to be an interaction between the two factors.

Results

Paper I

The possible roles of cathepsin B and recombinant mast cell β 1-tryptase in the activation and degradation of procarboxypeptidase B, trypsinogen 1 and proelastase 2 were investigated in this paper.

Activation and degradation of pancreatic proenzymes by cathepsin B

Cathepsin B activated trypsinogen, but after 3 hours of incubation, the activation curve leveled out at a trypsin activity of about 30% of the corresponding activity observed after activation by enterokinase. There was a discrepancy between trypsin activity and TAP concentration in samples of trypsinogen activated by cathepsin B. The concentration of TAP after incubation with cathepsin B merely reached 10% of TAP concentration seen in enterokinase activated samples. Based on these results it was hypothesized that cathepsin B can degrade TAP. The possible degradation of TAP by cathepsin B was further investigated in a separate experiment. Pure synthetic TAP was incubated with cathepsin B and increasing concentrations of the cathepsin B inhibitor E64d. A rapid degradation of TAP was documented and this process was inhibited by E64d.

Inactivation of trypsinogen by cathepsin B was examined by addition of enterokinase to samples of trypsinogen incubated with cathepsin B. Trypsin activity was raised to con-

trol levels after addition of enterokinase, indicating that cathepsin B had not degraded or inactivated trypsinogen to any significant extent. There was no sign of cathepsin B activation or degradation of procarboxypeptidase B or proelastase.

Activation of pancreatic proenzymes by monomeric and tetrameric trypsin

Neither tetrameric nor monomeric recombinant human β I-trypsin, caused any detectable activation of trypsinogen, procarboxypeptidase B or proelastase. Control experiments showed that all proenzymes could be activated by incubation in the presence of their corresponding physiological activating agents (enterokinase or trypsin).

Paper II

Trends in the incidence of acute pancreatitis related to different risk factors are reported in this paper.

Cases of acute pancreatitis in Malmö 1985–1999

A computer search for the diagnostic code of acute pancreatitis in clinical, autopsy and forensic records gave 1444 hits during the investigated period. From this material, 929 correctly diagnosed first attacks of acute pancreatitis were identified. The most important associated risk factor was gallstone disease, identified in 392 cases (42.2%), followed by alcohol abuse, which was judged to be associated with the attack of acute pancreatitis in 228 cases (24.5%). The mean age of all cases of acute pancreatitis was 59.4 years. The mean age was 65.3 years in gallstone associated cases, 47.6 years in alcohol associated cases and 60.5 years in cases related to other or unknown risk factors.

Trends in incidence

Trends in incidence of first attacks of acute pancreatitis related to the main risk factor groups are presented in table 2. A marked, and statistically significant, increase in the incidence of gallstone related acute pancreatitis was found in both men and women.

There was a statistically significant decrease in alcohol-related acute pancreatitis among men, but no statistically significant trend among women. Acute pancreatitis related to other or unknown risk factors increased in both sexes, and this increase was statistically significant. The case mortality rate and the proportion of severe cases according to the Atlanta classification (6) was similar throughout the investigated period. There was no statistically significant trend in mean age of patients with gallstone-related acute pancreatitis (0.0 years per year, 95% CI -0.6 to 0.2) or patients with an attack of acute pancreatitis related to unknown or other risk factors (-0.1 years per year, -0.5 to 0.3). The mean age of patients with alcohol-related acute pancreatitis increased (0.4 years per year, 0.1 to 0.7).

Correlation to underlying risk factors in the background population

The incidence of gallstone disease in the background population increased in both men (1.8% per year (95% CI 0.2 to 3.4)) and women (1.9%, 0.1 to 3.7). These trends were correlated to the incidence of gallstone-related acute pancreatitis in men ($p = 0.007$) and women ($p = 0.026$) respectively.

Among men there was a decrease in mortality from cirrhosis (-4.3%, -6.7 to -1.9) and delirium tremens (-13%, -17.0 to -9.3). Trends in the incidence of alcohol-related acute pancreatitis were correlated to mortality from cirrhosis ($p = 0.02$) and delirium tremens ($p = 0.014$), among men. Mortality from cirrhosis decreased among women (-5.7%, -11

Table 2. Results paper II.

Annual change in age standardized incidence of acute pancreatitis.

	All		Men		Women	
	r (p)	Annual change in % (95% CI*)	r (p)	Annual change in % (95% CI*)	r (p)	Annual change in % (95% CI*)
All pancreatitis	0.81 (<0.001)	3.9 (2.1 to 5.8)	0.36 (0.19)	1.8 (-0.8 to 4.5)	0.73 (0.002)	6.5 (2.7 to 10.4)
Alcohol associated pancreatitis	-0.77 (0.001)	-5.1 (-7.4 to -2.8)	-0.68 (0.006)	-5.5 (-8.7 to -2.2)	-0.34 (0.22)	-2.1 (-8.1 to 4.3)
Gallstone associated pancreatitis	0.78 (0.001)	7.6 (4.0 to 11.4)	0.73 (0.002)	7.4 (2.8 to 12.2)	0.68 (0.005)	8.0 (1.9 to 14.5)
Other or unknown pancreatitis	0.85 (<0.001)	5.8 (3.5 to 8.1)	0.54 (0.039)	3.8 (0.3 to 7.5)	0.68 (0.005)	7.5 (1.8 to 13.5)

*CI, confidence interval

to -0.6), but there was no significant trend in delirium tremens (-5.5%, -14 to 4.4). None of these conditions showed any statistically significant correlation with alcohol-related acute pancreatitis among women. The incidence of lung cancer decreased among men (-4%, -5.4 to -2.7) and increased among women (2.6%, 1.2 to 4.0) in the background population. The incidence in alcohol-related acute pancreatitis was correlated to the incidence of lung cancer among men ($p = 0.006$) but not among women ($p = 0.34$).

Paper III

Risk factors for acute pancreatitis were investigated in this paper. Baseline data from the Malmö Preventive Project was used for exposure assessment. Cases of acute pancreatitis were compared to the rest of the cohort.

Smoking and acute pancreatitis

Current smoking was associated with acute pancreatitis after adjustment for age, sex, BMI, and Mm-MAST category (table 3).

A dose-response relationship was revealed and the strongest association was seen in smokers of 20–30 cigarettes per day. The RR for acute pancreatitis was not increased in former smokers. Using γ -GT quartiles instead of Mm-MAST as indicator for alcohol consumption gave similar risk estimates. Current smoking increased the RR for acute pancreatitis in every stratum of Mm-MAST category, and in all γ -GT quartiles. No significant interaction was detected between smoking status and Mm-MAST ($p = 0.48$) category, or smoking status and γ -GT quartile ($p = 0.66$).

The association between smoking and

acute pancreatitis was further investigated in different etiological groups of acute pancreatitis. The RRs among current smokers (adjusted for age, sex, BMI and Mm-MAST) were 1.51 (95% CI 0.81 to 2.82) for gallstone-related acute pancreatitis, 2.94 (1.42 to 6.05) for alcohol-related acute pancreatitis and 2.35

(1.27 to 4.37) for pancreatitis related to other or unknown risk factors.

Other risk factors

Alcohol consumption was associated with acute pancreatitis in a dose-response manner

Table 3. Results paper III.

Relative risk for acute pancreatitis adjusted for age, sex, body mass index, smoking status and alcohol consumption.

Factor	Category	Relative Risk (95% confidence interval)	p-value
Smoking status and tobacco dose	Never	ref	ref
	All Current	2.14 (1.48 to 3.09)	<0.001
	<20 cigarettes/day	1.84 (1.19 to 2.85)	0.006
	20–30 cigarettes/day	3.19 (2.03 to 5.00)	<0.001
	>30 cigarettes/day	2.87 (1.57 to 5.24)	0.001
	Missing dose	1.38 (0.77 to 2.46)	0.28
	Former	1.09 (0.66 to 1.80)	0.74
	Missing status	–	–
	Trend (never→>30/day)	–	<0.001
γ-GT*-quartile (μkat/L)	1 (<0.29)	ref	ref
	2 (0.29–0.41)	1.05 (0.62 to 1.80)	0.84
	3 (0.41–0.63)	1.45 (0.88 to 2.38)	0.15
	4 (>0.63)	2.14 (1.32 to 3.49)	0.002
	Missing	3.11 (0.42 to 22.9)	0.26
	Trend (multiples of 0.1)	2.45 (1.39 to 4.33)	0.002
Alcohol consumption (Mm-MAST** category)	Low	ref	ref
	Intermediate	1.21 (0.85 to 1.72)	0.30
	High	2.55 (1.59 to 4.08)	<0.001
	Missing	1.00 (0.57 to 1.77)	0.99
	Trend (low–high)	–	<0.001
Body mass index (kg/m ²)	<20	1.02 (0.56 to 1.88)	0.95
	20–25	ref	ref
	25–30	1.15 (0.83 to 1.60)	0.39
	30<	1.45 (0.85 to 2.48)	0.18
	Missing	–	–
	Trend (continuous)	1.05 (1.01 to 1.09)	0.02

*γ-GT, gamma glutamyl transferase

** Malmö modification of the brief Michigan alcoholism screening test

after adjustment for age, sex, BMI and smoking. This association was observed for both methods of estimating alcohol consumption (γ -GT or Mm-MAST) (table 3). There was no significant increase in RR for acute pancreatitis in any BMI category. However, when BMI was entered as a continuous variable, a weak, but significant association, to the risk for acute pancreatitis was observed (table 3).

Paper IV

The consequences of long term nicotine exposure on exocrine pancreas were studied in this paper. Concentrations and production of pancreatic proenzymes were investigated in pancreatic extracts and in plasma. Two new RIAs for analysis of TAP 1 and TAP 2 in the rat were developed.

Radioimmunoassays for the activation peptides of trypsinogen 1 and 2

The TAP 1 antibody did not precipitate TAP 2 to any great extent (<10%). The TAP 2 antibody was less specific, precipitating synthetic TAP 1 to 30–40%. However, the importance of this cross-reaction was lower than expected when samples of activated trypsinogens were investigated by preparative electrophoresis of a rat pancreatic extract. The assays were capable of detecting concentrations of the peptides as low as 0.1 nM. Boiling the peptides for 15 minutes did not affect immunoreactivity.

Effect of long term nicotine exposure

Weight gain was reduced in nicotine treated animals, mainly due to a decrease in weight the first week after introduction of nicotine in the drinking water. Concentrations of different proenzymes for nicotine treated animals and controls are illustrated in figure 6. All given concentrations are ratios of the crude con-

centration over DNA concentration. This was done to give concentrations per cell in order to compensate for interstitial differences (i.e. edema or fibrosis) between the groups. Both trypsinogen 1 and 2 were elevated in nicotine treated animals and there was a significant increase in the ratio of trypsinogen 2/1. Amylase activity was increased in pancreatic extracts but not in plasma after nicotine exposure.

In situ hybridization for mRNA for trypsinogen 1, trypsinogen 2, procolipase and the CCK-A receptor did not reveal any differences between the groups.

General discussion

During the last century considerable effort has been made to describe and understand the causes and initiating events of acute pancreatitis. Epidemiological observations by clinicians and experimental work have made valuable contributions to the understanding of the disease. Careful observations established the principal risk factors, gallstone disease and alcohol abuse, already in the late 19th century (2). Autopsy findings of massive local necrosis aroused the curiosity of early investigators and prompted the theory of autodigestion which has been investigated in numerous animal studies. The main aim of this work was to further investigate the initiation of the cascade of events that eventually leads to acute pancreatitis. Both epidemiology and biochemistry have been tools for this research.

The role of cathepsin B and mast cell tryptase in acute pancreatitis

Most investigators agree today that premature activation of the pancreatic proenzymes is a crucial event in the development of acute pancreatitis. However, no consensus has been reached on when, where and how this activation takes place. The theory that has gained

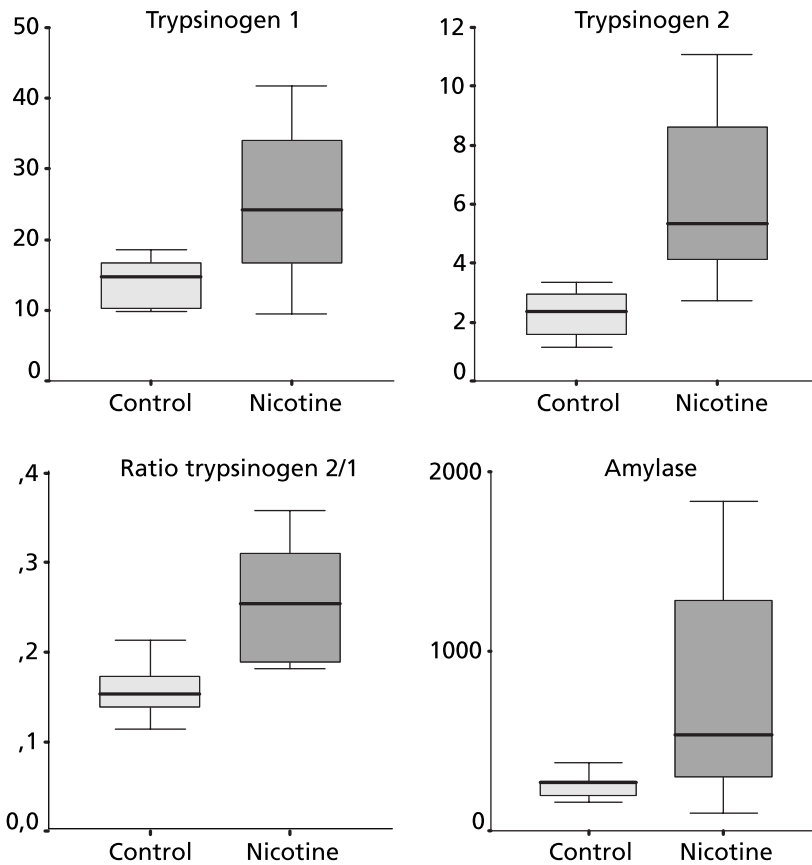


Figure 6. Results paper IV.

Trypsinogen 1 and 2 concentrations were indirectly measured by activation of pancreatic extracts with enterokinase and subsequent quantification of their respective activation peptide by radioimmunoassay. Amylase was measured by activity towards 4,6-ethyliden-G7-p-nitrofenol. All concentrations are expressed as the ratio of the crude concentration over the DNA concentration in the sample.

most acceptance during the last decades is that cathepsin B is responsible for this activation after co-localization of lysosomes and secretory granules (63). The ability of cathepsin B to activate trypsinogen has been known since the late 1950s (76), and direct activation of pancreatic proenzymes other than trypsinogen has also been proposed.

In paper I, cathepsin B was demonstrated to activate trypsinogen, but the process was not as efficient as activation by enterokinase. The yield of trypsin activity after incubation with cathepsin B was about 30% of that seen

with enterokinase. A possible explanation to this, previously suggested by Greenbaum *et al.*, is that trypsin (or trypsinogen) is degraded by cathepsin B (76). However, when enterokinase was added to samples incubated with cathepsin B, trypsin activity increased to levels similar to those seen in samples that were incubated with enterokinase alone. TAP concentration after incubation with cathepsin B was markedly lower than what could be expected from trypsin activity. This finding gave rise to the suspicion that cathepsin B might degrade TAP. This hypothesis was confirmed in a sep-

arate experiment where the concentration of TAP was demonstrated to decrease rapidly in the presence of active cathepsin B. Alternative explanations for the incomplete activation of trypsinogen by cathepsin B could be proposed. In the light of the observed degradation of TAP mediated by cathepsin B it is tempting to speculate that not all eight amino acids of the TAP are cleaved off when trypsinogen is activated by cathepsin B. This would explain why addition of enterokinase can augment trypsin activity in cathepsin B activated samples. However, additional studies are needed to further investigate this issue. Cathepsin B did not activate procarboxypeptidase B or proelastase. We consider our findings compatible with the co-localization theory. If cathepsin B plays a role in the activation of pancreatic proenzymes during the early course of acute pancreatitis, this occurs probably through activation of trypsinogen to trypsin. Given the acidic pH-optimum of cathepsin B, this is probably an intracellular process (65).

Mast cell tryptase does not seem to be of importance in the activation of pancreatic proenzymes. As demonstrated in paper I, recombinant β I-tryptase did not activate any of the investigated enzymes, neither in its tetrameric nor in its monomeric form.

Time trends in the incidence of acute pancreatitis

Several studies have shown increasing incidence of acute pancreatitis during the last decades (108–110, 112, 116, 118–123). Proposed explanations have been changing prevalence of underlying risk factors and improved diagnosis (108–110, 116, 119, 122, 123, 126, 127). In paper II, trends in the incidence of first attacks of acute pancreatitis in Malmö were investigated. Increasing incidence of gallstone-related acute pancreatitis was demonstrated for both sexes. The incidence of alcohol-related acute pancreatitis was decreasing for men, but there was no significant trend for women.

Methodological issues

Definition of the population at risk and case retrieval are crucial tasks in an incidence study. Erroneous results could be obtained due to loss of cases or improper inclusion of cases from outside the study population. This type of bias is usually referred to as selection bias. Referral of patients to or from other hospitals is a common source of selection bias which can lead to both over- and underestimation of incidence rates. In Malmö the definition of the population at risk and the question of case retrieval were facilitated by the fact that there is only one hospital for somatic diseases in Malmö without any referrals of patients with acute pancreatitis to or from this hospital.

Detection bias is another possible source of error. An example of this is when diagnostic tools for the detection of a disease are improved or altered. An expected consequence of lowering the diagnostic threshold for a disease is that the proportion of mild cases will increase and the case fatality rate will decrease. No such trend was observed in paper II, indicating that the risk for this type of detection bias was probably low.

Misclassification bias can also give misleading results. Misclassifications may have occurred both when diagnostic criteria for acute pancreatitis were revised and when plausible etiology was assessed. To lower the risk for misclassification bias, case files were reviewed by only two investigators working in close contact and using a standard protocol. Only first attacks of acute pancreatitis were included. Assessment of etiology, and especially differentiation between alcohol-related acute pancreatitis and pancreatitis of unknown etiology, is associated with a risk for detection bias in the retrospective setting. Due to the retrospective design of the study, data on exact amounts of ingested alcohol was not available for most cases. The mean age for cases with alcohol-related pancreatitis and pancreatitis of unknown/other etiology differ markedly

(47.6 years and 60.5 years, respectively in paper II). If the decrease in alcohol-related acute pancreatitis were to be explainable by an increasing tendency over time to classify cases with only weak evidence of alcohol abuse as of unknown etiology, a decreasing mean age in the group of unknown cases would be expected. However, in paper II there was no significant trend in mean age of the group with acute pancreatitis related to unknown or other risk factors. We therefore consider a substantial misclassification bias of etiology in paper II to be unlikely.

Trends in the incidence of acute pancreatitis

Gallstone-related acute pancreatitis increased in both men and women and these trends were correlated to increasing trends in the incidence of other gallstone-related conditions in the background population. There was no significant trend in the mean age of patients with gallstone-related acute pancreatitis suggesting that this group was constituted by the same type of patients throughout the period. Increasing incidence of gallstone-related acute pancreatitis has been suggested by other studies (110). The prevalence of overweight, a known risk factor for gallstone disease (165), has also been demonstrated to increase in Malmö during the investigated period (166). Hence, an increasing prevalence of gallstones in the population at risk probably contributes to the observed increasing incidence of gallstone-related acute pancreatitis. In addition, despite the increasing incidence of gallstone disease, local register did not indicate any increase in the frequency of cholecystectomies performed at Malmö University Hospital. This might also have influenced the incidence of gallstone-related acute pancreatitis.

The incidence of alcohol-related acute pancreatitis decreased among men but there was no significant trend among women. Trends in markers for alcohol consumption in the

background population were investigated in order to search for possible explanations to this somewhat unexpected finding. The decreasing incidence of alcohol-related acute pancreatitis among men was correlated to a decrease in both the mortality from cirrhosis and the incidence of delirium tremens, suggesting a decrease in alcohol consumption in the background population. The proportion of residents in Malmö with a foreign background has increased during the investigated period (164). Some of these immigrants come from cultures where alcohol consumption is lower than in the native Swedish population. This might be a contributing explanation to a potentially decreased incidence of alcohol related disease. However, official statistics on sales of alcohol do not indicate any decrease in alcohol consumption on a national level (156) and there are no reliable local statistics on alcohol consumption. Hence, it is difficult to conclude whether the consumption of alcohol have decreased or increased in Malmö during the studied period and this will have to be further investigated.

An alternative explanation to the decreased incidence of alcohol related acute pancreatitis could be the existence of an additional risk factor. From the observed trends in acute pancreatitis it can be postulated that this risk factor should decrease among men and be fairly stable among women and that it probably affects alcohol-related pancreatitis more than gallstone-related pancreatitis. Smoking has been shown to impair pancreatic exocrine function. The association between alcohol-related acute pancreatitis and smoking-related disease was therefore investigated. The incidence of alcohol-related pancreatitis was significantly correlated to the incidence of lung cancer among men but not among women. Official statistics on smoking habits on a national basis have demonstrated a marked decrease in the proportion of smokers among men but not among women during the investigated period in the age group with the highest prevalence of

acute pancreatitis (155). Hence, it is possible that changes in smoking habits have contributed to the observed trends in alcohol-related acute pancreatitis. A limitation of this finding is a potential ecological fallacy as we do not know if smoking individuals in the population were those who got acute pancreatitis to a greater extent. As a consequence, this observation does not allow for conclusions about causality and should be regarded as hypothesis generating. The possible association between smoking and acute pancreatitis was further studied in paper III and IV.

BMI and acute pancreatitis

There was no significant increase in the relative risk (RR) of acute pancreatitis in any of the investigated BMI categories. However, a weak but significant association was demonstrated when BMI was entered as a continuous variable. No information was gathered on the prevalence of gallstone disease collected at baseline investigation. Since overweight is a known risk factor for gallstone disease (165) it is possible that the association between BMI and acute pancreatitis is mediated by an increased prevalence of gallstones in overweight subjects.

Smoking and acute pancreatitis

Smoking is an established risk factor for both pancreatic cancer (133) and chronic pancreatitis (11, 12, 134, 135). In paper II a correlation between the incidence of alcohol-related acute pancreatitis and mortality from lung cancer was observed. This directed our interest towards a possible association between acute pancreatitis and smoking. Some indications already exist for such an association (134, 139, 140). In a cohort study (paper III) we investigated the association between acute pancreatitis and the possible risk factors age, sex, BMI, alcohol consumption and smoking.

A true association or confounding by alcohol?

Controlling for alcohol consumption is crucial when studying the association between smoking and acute pancreatitis. Alcohol abuse is a known risk factor for acute pancreatitis, and alcohol consumption and smoking are known to be associated (138, 167). Two separate parameters for estimation of alcohol consumption were available in the Malmö Preventive Project cohort: self reported pattern of alcohol consumption (Mm-MAST) and γ -GT value. Mm-MAST is a validated screening tool for alcoholism, directed at detecting individuals with a pattern of alcohol consumption that is in the risk zone of alcohol addiction. The advantage with this test is that it probably can give more valid information on drinking habits than direct questions on amounts of ingested alcohol. A disadvantage with this test is that it does not produce quantitative data. Blood levels of γ -GT is relatively sensitive for high alcohol consumption but the test's specificity is low (168). A number of different drugs and conditions (e.g. obesity and hepatic diseases) are also associated with increased γ -GT values (169). The relatively high sensitivity of γ -GT implies that it may be useful for identifying low consumers of alcohol. The validity of the smoking variable in this cohort has been investigated previously, and has been demonstrated to be correlated to plasma levels of carboxyhemoglobin (170).

Smoking was associated with the risk for acute pancreatitis after adjustment for age, sex, BMI and alcohol consumption. This result was independent of using either Mm-MAST or γ -GT to estimate alcohol consumption. The association between smoking and acute pancreatitis was further studied in separate strata of alcohol consumption as measured by both Mm-MAST and γ -GT. The association between smoking and acute pancreatitis was consistent in all strata of alcohol consumption. No statistically significant interaction was dem-

onstrated between smoking and Mm-MAST or between smoking and γ -GT. Considering the above-mentioned analyses, we believe that we have been able to separate the association between smoking and acute pancreatitis from the effect of alcohol consumption.

Alcohol-related acute pancreatitis was the type of pancreatitis that was most firmly associated with smoking followed by idiopathic acute pancreatitis. The RR for gallstone-related acute pancreatitis was slightly but not statistically significantly increased among current smokers. These results are well in accordance with what has previously been published by Morton *et al.* (140).

Effect of nicotine and smoking on exocrine pancreas

The influence of nicotine on concentration and production of pancreatic proenzymes was studied in paper IV. Two new RIAs for quantification of TAP 1 and 2 respectively were presented in this paper. The specificity of the TAP 1 RIA was satisfactory, but there was a considerable cross-reaction of the synthetic TAP 1 peptide in the TAP 2 RIA. However, the importance of this cross-reaction was surprisingly moderate when TAP 2 was measured in samples of activated trypsinogen. A possible explanation to this could be that the immunoreactivity of TAP 2 is altered after cleavage from trypsinogen by further degradation mediated by some other active enzyme in the sample. This was shown to actually occur for human TAP in paper I where cathepsin B was demonstrated to degrade immunoreactive TAP. As a consequence of the cross-reaction in the TAP 2 RIA, trypsinogen 2 concentrations and absolute ratios of trypsinogen 2/1 might be overestimated in paper IV and must be interpreted cautiously. However, observed changes in the ratio of trypsinogen 2/1 are probably reliable since this cross-reaction can be assumed to be constant throughout all experiments.

Weight gain was reduced in nicotine treated animals. This was an expected effect of nicotine administration through drinking water that has been described previously (14). It is not excluded that the difference in weight gain influenced the results of paper IV. However, increased intracellular concentration of pancreatic proenzymes is not an expected consequence of starvation or food restriction. On the contrary, previous studies have demonstrated depletion of pancreatic proenzymes after starvation (171) and unaffected amylase concentration after 10% food restriction (172). Therefore it seems unlikely, that the reduced weight gain in itself would cause the increased concentrations of proenzymes seen in nicotine treated animals in this study.

Concentrations of amylase, trypsinogen 1 and trypsinogen 2 were increased in pancreatic extracts in nicotine treated animals, in accordance with previous studies (14, 145, 152). The ratio of trypsinogen 2 over trypsinogen 1 was increased in the nicotine treated group. The effect of nicotine exposure on this ratio has not been investigated previously but an increased ratio of trypsinogen 2/1 have been described in different kinds of pancreatic disease, including pancreatic cancer, pancreatitis and rejection of pancreatic transplants (173–176). Hyperstimulation with CCK has also been shown to increase this ratio both on protein and mRNA level (177, 178). Whether the increased ratio of trypsinogen 2/1 observed in paper IV was a result of a primary effect of nicotine or secondary due to an increased CCK stimulation is unknown since CCK was not measured. Previous investigators have demonstrated that nicotine exposure causes decreased responsiveness to CCK-stimulation by the acinar cell but does not impact CCK membrane binding capacity or membrane dissociation constant. On the basis of these findings it has been hypothesized that the inhibitory effect of nicotine on CCK-stimulated acinar cell secretion is exerted by a post-receptor mechanism. In accordance with this hypothesis, our results

indicated no difference in the expression of mRNA for the CCKA receptor between the two groups.

An increase in the concentration of proenzymes in pancreatic extracts could theoretically be due either to increased production or decreased secretion of the proenzymes. Production of amylase has been investigated previously in the rat after nicotine exposure and these studies suggest that the production of amylase is increased in nicotine exposed animals (145, 152, 153). However our results in paper IV did not reveal any signs of an increased production of procolipase, trypsinogen 1 or trypsinogen 2 in nicotine treated animals, as shown by *in situ* hybridization. We do not see any obvious explanation for this discrepancy. It is not excluded that nicotine affects the production of the investigated enzymes in different ways. The administration route and dose of nicotine might also be of importance.

Taken together, the observed increase in the concentration of pancreatic proenzymes in pancreatic extracts in combination with the absence of a corresponding increase in the production of the same enzymes, indicates that nicotine induces an impairment of acinar cell secretion. This might explain why the association between smoking and alcohol-related acute pancreatitis was found to be stronger than the association between smoking and gallstone-related pancreatitis in paper III. Alcohol is thought to cause pancreatitis by interference with acinar cell secretion and it is therefore plausible that the consequences of nicotine and alcohol can potentiate each other.

Does smoking cause acute pancreatitis?

During the 1960s when the association between smoking and lung cancer became apparent, the U.S. Surgeon General appointed an expert committee to review the evidence. The committee developed a set of guidelines,

consisting of nine points for judging whether the association of a factor with a disease is causal (179). The evidence for an association between smoking and acute pancreatitis are reviewed according to these guidelines in box 1. (140, 180–185).

One point that has been questioned is number 6, whether there is a confounding by alcohol or not. Arguments for smoking as a risk factor for acute pancreatitis independent from alcohol consumption have been presented in this thesis. Further studies on the consequences of smoking on pancreatic function and pathology on a molecular level are warranted to increase the understanding of the relationship between smoking and pancreatic disease.

Conclusions

From studies presented in this thesis it is concluded that:

- Human cathepsin B can activate trypsinogen but not proelastase or procarboxypeptidase B. If cathepsin B is to play a role in the activation of pancreatic proenzymes during acute pancreatitis, this probably occurs by intracellular activation of trypsinogen followed by subsequent activation of the other proenzymes by active trypsin.
- Human mast cell tryptase can not activate trypsinogen, proelastase or procarboxypeptidase B.
- The incidence of gallstone-related acute pancreatitis increased in Malmö 1985–1999 among both men and women and this trend was correlated to an increasing incidence of other gallstone-related conditions in the same population.
- The incidence for alcohol-related acute pancreatitis decreased among men in Malmö 1985–1999 and this trend was correlated to a decreasing incidence of other conditions related to alcohol abuse, as well as to

Box 1. Evidence for a causal relationship between smoking and acute pancreatitis according to the guidelines of U.S. Surgeon General.

1. **Temporal relationship.** If an agent is to be regarded as causative the exposure has to precede the development of the disease. Such a temporal relationship between smoking and acute pancreatitis is demonstrated in paper III.
2. **Strength of the association.** The stronger the association, the more likely that the relationship is causal. The RR of acute pancreatitis among current smokers was 2.14 in the Malmö Preventive Project. This is below the RR of around 15–20 usually reported for lung cancer among smokers (180, 181), but similar to the generally accepted association between smoking and pancreatic cancer (RR around 2) (182) and even stronger than the equally accepted association between smoking and renal cancer (RR 1.3–2) (183).
3. **Dose-response relationship.** A dose-response relationship speaks in favor of causality. This type of association between smoking and the risk for acute pancreatitis was demonstrated in paper III and has previously been observed by other investigators (140).
4. **Replication of the findings.** A true causal relationship can be expected to be replicable and consistent within different subgroups of a population. To date there is, to our knowledge, two case-control studies and two cohort studies (including paper III) that have shown a relationship between smoking and acute pancreatitis. In paper III it is also demonstrated that the association between smoking and acute pancreatitis exists in all subgroups when the population is stratified for alcohol consumption.
5. **Biologic plausibility.** Causality is considered more likely if epidemiological observations are consistent with current biological knowledge. Biological evidence for a noxious influence of smoking and nicotine are reviewed in table 1 and data from paper IV further supports this notion.
6. **Consideration of alternative explanations.** Confounding by alcohol consumption is the main alternative explanation that has to be considered when reviewing the relationship between smoking and acute pancreatitis. In paper III, the association between smoking and the risk for acute pancreatitis and was consistent after adjustment for alcohol consumption in the Cox analysis, and similar in all strata of alcohol consumption. The interaction analysis did not reveal any significant interaction between smoking and alcohol. This suggests that confounding by alcohol can not explain the observed association between smoking and alcohol consumption.
7. **Cessation of exposure.** A decline in the risk for a disease is expected if a factor that is proposed to cause the disease is removed. In paper III, the RR of acute pancreatitis was lower in ex-smokers than in current smokers.
8. **Consistency with other knowledge.** A causative relationship can be expected to be consistent with other data in a population. For instance, if smoking is associated with acute pancreatitis, the incidence of the disease can be expected to be influenced by consumption of tobacco in a population. In paper II the incidence of alcohol-related acute pancreatitis was demonstrated to correlate with the mortality from lung cancer and national trends in the proportion of smokers are parallel to incidence trends in alcohol-related acute pancreatitis in the study population.
9. **Specificity of the association.** In the first guidelines it was stated that if one agent was associated with only one disease, a casual relationship was more likely. This guideline has been questioned and is nowadays not given any major consideration (184). For example smoking has been firmly associated with several conditions including heart disease, lung cancer, bladder cancer, renal cancer and pancreatic cancer (185).

a decreasing incidence of lung cancer, in the same population. There was no statistically significant trend in the incidence of alcohol-related acute pancreatitis among women.

- Smoking is associated with acute pancreatitis in a dose-response manner after adjustment for age, sex, BMI and alcohol consumption. There is a weak association be-

tween BMI and the risk for acute pancreatitis.

- Long term nicotine exposure in the rat leads to increased concentrations of pancreatic proenzymes in pancreatic extracts but has no impact on corresponding mRNA levels. These results suggest that nicotine impairs secretion rather than production of pancreatic proenzymes.

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Populärvetenskaplig sammanfattning

I denna avhandling redovisas fyra delstudier som alla handlar om akut inflammation i bukspottskörteln.

Bakgrund och målsättning

Bukspottskörteln har två separata huvudfunktioner: att producera de hormoner som styr energiomsättningen i kroppen samt att utsöndra bukspott. Bukspottet innehåller ett antal olika enzymer som tillsammans bryter ner proteiner, fetter, kolhydrater och andra beståndsdelar i födan. Eftersom detta är samma beståndsdelar som bygger upp vår egen kropp måste kroppens egna vävnader skyddas från bukspottets inverkan. Bukspottets enzymer utsöndras därför i inaktiv form och aktiveras först när de når tolvfingertarmen. Detta sker genom att ett av bukspottets enzym, trypsinogen, aktiveras till trypsin av ett enzym som finns i tarmväggen. Trypsin aktiverar sedan de övriga enzymerna. Tarmväggen tar inte skada av bukspottets enzymer eftersom den kläds av ett skyddande slemskikt.

I vissa fall kan det hända att bukspottets enzymer aktiveras alltför tidigt, redan inne i bukspottskörteln. Man tror att det är detta som är den utlösande faktorn vid akut inflam-

mation i bukspottskörteln. Vad som orsakar denna oönskade aktivering av bukspottets enzymer är dock inte klarlagt. Cathepsin B är ett enzym som finns inne i alla kroppens celler. Cathepsin B har visats kunna aktivera trypsinogen och det har även föreslagits att cathepsin B skulle kunna aktivera övriga enzym i bukspottet. Ett annat enzym som skulle kunna stå för aktiveringen av trypsinogen vid akut inflammation i bukspottskörteln är tryptas som utsöndras från en typ av celler som tillhör immunförsvaret och kallas mastceller.

Akut inflammation i bukspottskörteln är en relativt vanlig sjukdom som i Malmö drabbar cirka 30 personer per 100 000 invånare och år. Man har rapporterat en ökande incidens (antal insjuknanden per år dividerat med antalet individer i befolkningen) i akut inflammation i bukspottskörteln från flera centra i världen de senaste åren. De viktigaste orsakerna till sjukdomen är förekomst av gallsten samt alkoholöverkonsumtion. I cirka 60–70% av alla fall av akut inflammation i bukspottskörteln kan någon av dessa två orsaker identifieras hos den drabbade individen. Andra mindre vanliga orsaker har även beskrivits men i cirka 20% av alla fall av inflammation i bukspottskörteln kan man inte identifiera någon sanno-

lik orsak. Vidare är det bara en bråkdel av de individer i befolkningen som har gallstenar eller som överkonsumerar alkohol som drabbas av akut inflammation i bukspottskörteln. Det finns därför sannolikt ytterligare faktorer som är av betydelse för risken att insjukna denna sjukdom. Rökning har tidigare visats vara kopplat till risken att utveckla cancer och kronisk inflammation i bukspottskörteln och det finns bevis från djurförsök att nikotin påverkar cellerna i bukspottskörteln.

Huvudsyftet med denna avhandling är att ytterligare undersöka hur akut inflammation i bukspottskörteln uppstår. Möjligheten att rökning skulle kunna vara en orsak för sjukdomen undersöks särskilt noga. Följande delmål sattes upp för att, på olika vis, belysa de mekanismer som leder till akut inflammation i bukspottskörteln:

- Att undersöka förmågan hos cathepsin B och tryptas från mastceller att aktivera enzymer i bukspottet.
- Att undersöka trender i incidensen av akut inflammation i bukspottskörteln i Malmö 1985–1999.
- Att undersöka om övervikt och rökning innebär en ökad risk för akut inflammation i bukspottskörteln.
- Att undersöka effekten av nikotin på bukspottskörteln hos råttor.

Genomförande och resultat

Trypsinogen och två andra enzymer från bukspottet (proelastas och procarboxypeptidas) renades fram från bukspott uppsamlat från en patient som genomgått en operation av bukspottskörteln. Cathepsin B respektive tryptas från mastceller tillsattes till de olika enzymerna. Cathepsin B aktiverade trypsinogen men inte proelastas eller procarboxypeptidas. Inget av enzymerna aktiverades av tryptas. Dessa resultat indikerar att tryptas sannolikt inte har någon betydelse för aktiveringen av bukspottets enzym vid akut inflammation i bukspottskörteln. Det är däremot möjligt att

cathepsin B är det enzym som utlöser denna aktivering. Eftersom trypsinogen förefaller vara det enda enzym som aktiveras av cathepsin B så är det sannolikt det aktiva trypsinet som sedan aktiverar de övriga enzymen i bukspottet.

Incidensen av akut inflammation i bukspottskörteln ökade med cirka 50% i Malmö från 1985 till 1999. Denna ökning bestod i en kraftig ökning av fall som var relaterade till gallstenssjukdom och sådana där ingen bakomliggande orsak gick att identifiera. Andra gallstensrelaterade sjukdomar ökade också i samma befolkning och denna trend samvarierade med ökningen i gallstensrelaterad inflammation i bukspottskörteln. Incidensen av alkoholrelaterad inflammation i bukspottskörteln sjönk hos män men ingen tydlig trend gick att utläsa hos kvinnor. Incidensen av andra tillstånd som är relaterade till hög alkoholkonsumtion (dödsfall i skrumplever och delirium tremens, ett tillstånd kopplat till alkohol abstinens) sjönk också bland män i befolkningen och dessa trender sammanföll med den sjunkande incidensen av alkoholrelaterad akut inflammation i bukspottskörteln hos män.

I Malmö genomfördes från mitten av 70-talet till början av 90-talet ett stort sjukdomsförebyggande projekt (Malmö förebyggande medicin) där en stor del av Malmös medelålders befolkning genomgick en hälsoundersökning främst för att undersöka faktorer som skulle kunna påverka risken för hjärt-kärlsjukdom. Information från dessa undersökningar finns lagrade i en databas och genom samkörning av det upprättade registret med alla insjuknanden i akut inflammation i bukspottskörteln 1985–1999 kunde 179 fall av akut inflammation i bukspottskörteln identifieras i Malmö förebyggande medicins databas. Dessa fall jämfördes med övriga individer i Malmö förebyggande medicin med avseende på ålder, kön, rökning, alkoholkonsumtion och övervikt i syfte att undersöka om dessa faktorer påverkade risken att insjukna i akut inflammation i bukspottskörteln. Med hjälp av statistisk databearbet-

ning kan effekten av varje enskild faktor isoleras. Det visade sig att rökning medförde en dubbling av risken för akut inflammation i bukspottskörteln, oberoende av hur mycket alkohol individen drack. Denna effekt var påvisbar även hos de individer som drack minst alkohol och risken ökade med antalet röka cigaretter per dag. Detta indikerar att det observerade sambandet mellan rökning och akut inflammation i bukspottskörteln sannolikt är verkligt och inte en konsekvens av att alkoholkonsumtionen hos rökare och icke-rökare skiljer sig åt. I detta material kunde även ett svagt samband mellan övervikt och risken att insjukna i akut inflammation i bukspottskörteln påvisas. Vidare kunde styrkan av det tidigare kända sambandet mellan alkoholkonsumtion och risken för sjukdomen mätas.

För att bättre förstå hur rökning kan leda till akut inflammation i bukspottskörteln genomfördes ett djurförsök där råttor exponerades för nikotin genom att en låg halt nikotin tillsattes till deras dricksvatten. Efter fyra veckor avlivades djuren. Man kunde då konstatera att koncentrationen av bukspottsenzym inne i bukspottskörteln var ökad hos de råttor som givits nikotinberikat vatten jämfört med kontrollråttor som hade druckit vanligt vatten. Analys av mRNA, som speglar produktionen av enzym i cellen, visade att produktionen av bukspottsenzym var lika stor hos nikotinbehandlade djur som hos kontroller. Kombinationen av ökad koncentration av enzym utan tecken till ökad produktion tyder på att nikotin orsakar en störning av utsön-

dringen av dessa enzym. En liknande störning i utsöndringen av bukspottsenzym har påvisats i djurmodeller av akut inflammation i bukspottskörteln. Det är möjligt att denna störning av förmågan att utsöndra enzym hos bukspottskörtelns celler och den resulterande ökade koncentrationen av potentiellt skadliga bukspottsenzym inne i körteln, är av betydelse för det påvisade sambandet mellan rökning och akut inflammation i bukspottskörteln.

Slutsatser

- Cathepsin B kan aktivera trypsinogen men inte de övriga enzymerna i bukspottet.
- Tryptas från mastceller kan inte aktivera de undersökta enzymerna i bukspottet.
- Incidensen av gallstensrelaterad inflammation i bukspottskörteln ökar, och incidensen alkoholrelaterad inflammation i bukspottskörteln minskar i Malmö.
- Rökning innebär en dubblad risk att drabbas av akut inflammation i bukspottskörteln oberoende av hur mycket alkohol man dricker.
- Övervikt medför en diskret ökad risk för akut inflammation i bukspottskörteln.
- Exponering för nikotin leder till ökad koncentration men inte ökad produktion av bukspottszymer i bukspottskörteln. Detta talar för att nikotin stör utsöndringen av enzym från bukspottskörteln vilket skulle kunna vara en bidragande orsak till varför rökning leder till ökad risk för sjukdom i bukspottskörteln.

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