Protease Activation and Inflammation in Acute Pancreatitis

Regnér, Sara

2008

Link to publication

Citation for published version (APA):

Total number of authors: 1

General rights
Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Protease Activation and Inflammation in Acute Pancreatitis

Sara Regnér
Abstract

Approximately 10–20% of patients with acute pancreatitis (AP) develop a severe disease with high mortality and morbidity. Activation of pancreatic proteases, the inflammatory response and impaired pancreatic circulation are pathophysiological events that are important in order for the disease to develop. There is no specific treatment for severe AP, and no useful marker for predicting the severity of the disease upon admission to the hospital.

In this thesis, markers of early pathophysiological events in AP are investigated, with emphasis on protease activation and inflammation.

Procarboxypeptidase B (proCAP) is a pancreatic proenzyme which, particularly in severe AP, is activated by trypsin thereby forming carboxypeptidase B (aCAP) and the activation peptide of procarboxypeptidase B (CAPAP).

An ELISA method for measurement of serum aCAP in patients with AP was developed, and aCAP was shown to inhibit fibrinolysis in vitro. This may contribute to formation of necrosis in AP. The prediction of severity and pathophysiology was studied in patients with mild (n=124) and severe (n=16) AP. Markers of protease activation (aCAP, CAPAP) and inflammation (monocyte chemoattractant protein-1 (MCP-1) and CRP) were found to be elevated within 24 hours in patients with severe AP. Protease activation decreased after 48 hours, yet inflammation persisted for a longer period of time. Markers of pancreatic leakage (proCAP) decreased with time without differences in patients with mild and severe AP. MCP-1 exhibited a good capacity at predicting severe AP upon hospital admission. CAPAP and aCAP may also be useful in predicting the degree of severity.

Key words:
Acute pancreatitis, carboxypeptidase B, aCAP, CAPAP, proCAP, MCP-1, fibrinolysis, prediction of severity
Alla långa sanningsrötter äro misstänkliga, sanningen får man endast ut i korta bitar.

*Edith Södergran*
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of papers</td>
<td>11</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>12</td>
</tr>
<tr>
<td>Introduction</td>
<td>13</td>
</tr>
<tr>
<td>Background</td>
<td>14</td>
</tr>
<tr>
<td>Exocrine pancreas</td>
<td>14</td>
</tr>
<tr>
<td>Protease activation in acute pancreatitis</td>
<td>14</td>
</tr>
<tr>
<td>Inflammatory changes in acute pancreatitis</td>
<td>17</td>
</tr>
<tr>
<td>Ischemia, microcirculation and microthrombosis</td>
<td>20</td>
</tr>
<tr>
<td>Fibrinolysis</td>
<td>21</td>
</tr>
<tr>
<td>Necrosis formation</td>
<td>22</td>
</tr>
<tr>
<td>Prediction of severity</td>
<td>22</td>
</tr>
<tr>
<td>Aims</td>
<td>25</td>
</tr>
<tr>
<td>Material, methods and results</td>
<td>25</td>
</tr>
<tr>
<td>Materials and methods, Paper I</td>
<td>25</td>
</tr>
<tr>
<td>Purification of aCAP</td>
<td>25</td>
</tr>
<tr>
<td>Antibodies</td>
<td>26</td>
</tr>
<tr>
<td>ELISA procedures</td>
<td>26</td>
</tr>
<tr>
<td>Analysis of proCAP and CAPAP</td>
<td>27</td>
</tr>
</tbody>
</table>
Results, Paper I ................................................................. 28
aCAP assay and cross reaction with proCAP ........................ 28
Normal serum .................................................................................. 28
Patient data ......................................................................................... 28
ir-aCAP in patient serum ................................................................. 29
Characterization of pure proCAP and aCAP by gel filtration ... 29
Ir-aCAP in relation to s-proCAP, u-CAPAP and maximum CRP levels ... 29
Recovery of ir-aCAP after gel filtration .......................................... 29
Interaction between aCAP and serum proteins ............................ 30
Materials and methods, Paper II and Paper III ......................... 30
Patients ......................................................................................... 30
Samples and assays ......................................................................... 30
Statistical methods ......................................................................... 31
Results, Paper II and III ............................................................... 31
Patient characteristics .................................................................... 31
Markers .......................................................................................... 31
Differences between mild and severe cases, Paper II ................. 31
Differences between mild and severe cases, Paper III ............... 34
Time course analysis ...................................................................... 35
Materials and methods, Paper IV ............................................... 35
aCAP ............................................................................................. 37
Fibrinolysis model: D-dimer measurements ................................. 37
Fibrinolysis and aCAP: D-dimer measurements ......................... 38
Agarose fibrinogen gels .................................................................. 38
Statistical methods ......................................................................... 38
Results, Paper IV .......................................................................... 39
Fibrinolysis model: D-dimer measurements ................................. 39
Fibrinolysis and aCAP: D-dimer measurements ......................... 39
Agarose fibrinogen gels .................................................................. 40
General discussion ......................................................................... 41
Conclusions ................................................................................................................................................................................................. 45

Implications for future studies and treatment of acute pancreatitis .......... 45

Acknowledgements ................................................................................................................................................................. 46

Populärvetenskaplig sammanfattning på svenska ........................................ 47

References ......................................................................................................................................................................................... 50

Papers

Paper I ................................................................................................................................................................................................................. 61

Paper II ........................................................................................................................................................................................................ 71

Paper III ....................................................................................................................................................................................................... 85

Paper IV .................................................................................................................................................................................................. 99
List of papers

This thesis is based on the following published papers, which will be referred to in the text by their Roman numerals. The papers are appended at the end of the thesis.

I. Borgström A, Regnér S
   Active Carboxypeptidase B Is Present in Free Form in Serum from Patients with Acute Pancreatitis.
   Pancreatology 2005;5:530–536. *

II. Regnér S, Appelros S, Hjalmarsson C, Manjer J, Sadic J and Borgström A
   Monocyte chemoattractant protein 1, active carboxypeptidase B and CAPAP at hospital admission are predictive markers for severe acute pancreatitis.
   Pancreatology 2008;00:00–00, in press. *

    Protease activation, pancreatic leakage and inflammation in acute pancreatitis: Differences between mild and severe cases and changes over the first three days
    Submitted.

IV. Regnér S, Borgström A†, Malm J, Manjer J and Appelros S.
    Active pancreatic carboxypeptidase B (aCAP) inhibits fibrinolysis in vitro: A potential mechanism behind necrosis formation in acute pancreatitis.
    Manuscript

† The authors wish to acknowledge with appreciation the contribution made to this papers by Professor Anders Borgström, who passed away on the 9th of October 2007.

* Reprinted from Pancreatology with permission from S.Karger AG, Basel.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-1PI</td>
<td>α-1 protease inhibitor</td>
</tr>
<tr>
<td>α₂-M</td>
<td>α₂ makroglobulin</td>
</tr>
<tr>
<td>AP</td>
<td>acute pancreatitis</td>
</tr>
<tr>
<td>APACHE</td>
<td>acute physiologic and chronic health evaluation</td>
</tr>
<tr>
<td>aCAP</td>
<td>active carboxypeptidase B</td>
</tr>
<tr>
<td>CAPAP</td>
<td>procarboxypeptidase B activation peptide</td>
</tr>
<tr>
<td>CCK</td>
<td>cholecystokinin</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CRP</td>
<td>c-reactive protein</td>
</tr>
<tr>
<td>CT</td>
<td>computerized tomography</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxyl terminal</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>E-selectin</td>
<td>endothelial selectin</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>ICU</td>
<td>intensive care unit</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL 1-ra</td>
<td>interleukin 1 receptor antagonist</td>
</tr>
<tr>
<td>Ir</td>
<td>immunoreactive</td>
</tr>
<tr>
<td>LR</td>
<td>likelihood ratio</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activation factor</td>
</tr>
<tr>
<td>PECAM</td>
<td>platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear</td>
</tr>
<tr>
<td>ProCAP</td>
<td>procarboxypeptidase B</td>
</tr>
<tr>
<td>P-selectin</td>
<td>platelet selectin</td>
</tr>
<tr>
<td>PSTI</td>
<td>pancreatic secretory trypsin inhibitor</td>
</tr>
<tr>
<td>PTC</td>
<td>procalcitonin</td>
</tr>
<tr>
<td>RER</td>
<td>rough endoplasmatic reticulum</td>
</tr>
<tr>
<td>ROC</td>
<td>receiver operating characteristics</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SIRS</td>
<td>systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SPINK1</td>
<td>serine protease inhibitor Kazal type 1</td>
</tr>
<tr>
<td>TAP</td>
<td>trypsinogen activation peptide</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue plasminogen activator</td>
</tr>
</tbody>
</table>
Introduction

In approximately 80–90% of patients with acute pancreatitis (AP), the course is mild and the disease self-limiting [1–3]. If systemic or local complications occur, the disease is classified as severe [4]. Mortality is high in this group, 17–43% and morbidity in terms of endocrine and/or exocrine pancreatic dysfunction or chronic pain is common [2,3,5,6].

The incidence of AP is reported to 5–73 /100 000 person-years. Higher incidence figures often represent studies where recurrent as well as first-time attacks of AP are included. Incidence also varies with the frequency of gallstone disease in the population and differences in alcohol consumption patterns, since these are the most important etiologic factors for AP [1–3,7–9].

AP is characterized by acute onset of severe abdominal pain and elevated levels of pancreatic enzymes in serum or urine. CT scan is used for diagnosis especially when other acute abdominal disorders are suspected or when levels of pancreatic enzymes are not elevated [10–13].

Pancreatic necrosis is a local complication frequently seen in severe AP. Infection of necrosis may lead to sepsis and death. Systemic inflammatory response syndrome (SIRS) is seen in patients with severe AP with or without pancreatic necrosis. The initial pancreatic inflammation has then become systemic and the pathophysiology in this phase of the disease resembles conditions such as sepsis, trauma and burns. Following SIRS, single or multiple organ dysfunctions may develop [14,15].

There is no specific therapy for patients with severe AP, but early monitoring, rehydration and supportive therapy, often in an intensive care unit (ICU) may lead to better prognosis [16]. In order to treat these patients in an optimal way, it is necessary to identify patients who will develop a severe disease early in the course. Clinical assessment at admission to hospital has a sensitivity for prediction of severe AP of approximately 40% [17,18]. Elevated levels of CRP correlates with severity, but since CRP reaches maximum levels 48–72 hours after onset of symptoms, it is not useful for prediction [19,20]. The lack of an early marker of severity is not only a problem in the clinical situation, but also for selecting patients to studies of treatment of severe AP. Since patients with mild AP recover completely without specific treatment, interpretations of results are difficult if these are included in studies. There are also ethical reasons not to test new treatments and risking unknown side effects for these patients, who would recover anyways.

The pathogenesis of AP is only partially known. Early in the course trypsinogen is prematurely activated to trypsin, which, in turn, activates other pancreatic proenzymes [21,22]. The exact impact of these proteases is not known, but autodigestion leading to pancreatic necrosis was proposed already more than 100 years ago [23]. The inflammatory reaction in AP is of importance later in the course of systemic disease as well as early in the pancreatic inflammatory phase [24–26]. Microcirculatory changes and ischemia are other factors contributing in the development of AP and the course of the disease [27–31]. Understanding of the pathophysiology, and especially the differences between mild and severe AP, is of importance when searching for treatments of severe AP. Knowledge about timing of the pathophysiological events is also of utmost importance for understanding when a treatment could be expected to be efficient.

Procarboxypeptidase B (proCAP) is a pancreatic proenzyme activated by trypsin early in the course of AP, leading to formation of active carboxypeptidase B (aCAP) and the activation peptide of procarboxypeptidase B (CAPAP) [32]. CAPAP has earlier
Protease activation and inflammation in acute pancreatitis

shown promising results as an early marker for prediction of severe AP, but its turnover in serum seems to be fast, which is probably due to its small molecular weight (10 kDa) [33,34]. The active enzyme, aCAP, is formed in equimolar amounts as CAPAP and should theoretically, from the perspective being larger (35 kDa), have a longer half life in serum. Hence, there is a theoretical background for aCAP to be an even better early marker of severe AP than CAPAP. As an active enzyme, aCAP could have biological effects of importance for the course of disease in AP. Other similar biological enzymes have been reported to interact with the fibrinolytic system and thereby inhibit fibrinolysis [35]. This may be important as inhibition of fibrinolysis in pancreas could theoretically contribute to pancreatic necrosis formed in severe AP.

This thesis examines early pathophysiological mechanisms in AP, especially focusing on protease activation and inflammation in the initiation and early phases of the disease. Markers for prediction of severity of AP, as well as the timecourses for markers of different pathophysiological events are studied. A method for measurement of aCAP is developed and its biological effect considering inhibition of fibrinolysis is investigated.

Background

Exocrine pancreas

Acinar cells in the exocrine pancreas are responsible for production of proteolytic, amylolytic and lipolytic enzymes needed for digestion of food. Bicarbonate is secreted from ductular cells, together with the enzymes. Secretion is regulated by neural as well as hormonal stimulation, mediated mainly by secretin for bicarbonate secretion and cholecystokinin (CCK) for enzyme release [36,37].

The acinar cell is usually protected from enzymatic damage by several mechanisms. Proteolytic enzymes, potentially capable of digesting cell membranes, are synthesized as proenzymes, (zymogens) without activity. Enzymes are kept inside membrane bound compartments; from their production site on the ribosomes attached to the rough endoplasmatic reticulum they migrate to the Golgi complex where digestive enzymes are packed in condensing vacuoles, maturing into zymogen granules before exocytosis from the apical side of the cell into the pancreatic duct, Figure 1. Trypsin inhibitors are synthesized and released together with the zymogens. Exocytosis is well regulated and stimulated by a rise in intracellular Ca²⁺ concentration [36,37], which is otherwise kept low, Figure 1. The low intracellular Ca²⁺ concentration favours degradation of trypsin rather than activation (see below). Another protective mechanism is the acidic pH inside the zymogen granules which precludes trypsin activity [38].

When pancreatic juice enters duodenum, trypsinogen is activated to trypsin by the brush border enzyme enterokinase in a reaction where the trypsinogen activation peptide (TAP) is cleaved off. Trypsin is responsible for activation of other proenzymes as well as of trypsinogen, yielding active proteases for digestion of food and corresponding activation peptides, such as CAPAP. Protease inhibitors located in the intestinal mucosa protects the mucosa from harmful effects of the proteases [36,37].

Protease activation in acute pancreatitis

In AP there is a premature activation of proteases and leakage of trypsinogen, other proenzymes, and activation peptides into the interstitium and blood are seen [19,34,39–46]. Elevated levels of trypsinogen and pro-CAP are seen in both mild and severe disease whereas levels of the activation peptides TAP
and CAPAP as well as trypsin in complex with α-1 protease inhibitor (α-1PI) are higher in severe AP [19,34,39–46]. The activation of trypsinogen to trypsin is an early event that might be the initiating step leading to AP. Theoretically this activation might occur inside acinar pancreatic cells, in the interstitium or in the pancreatic duct.

The possibility of intracellular activation is well documented in several studies on isolated acini cells and in experimental AP. According to the “co-localisation theory” trypsinogen activation occur inside acinar cells when zymogen granules confluences with other vacuoles, lysosomes, in the vacuolisation process. These changes are seen when apical exocytosis from acinar cells is blocked, Figure 1. Lysosomes contain hydrolases as Cathepsin B, which is able to activate trypsinogen and might play a role in the early activation process [22,47–53]. However, a colocalization of cathepsins with digestive enzymes has been observed in control animals under physiological conditions and even in healthy human pancreas trypsinogen and Cathepsin B were present in the same subcellular compartement [54]. Following this, there might be other factors or conditions affecting when and how intracellular trypsinogen activation occurs.

Interstitial activation of trypsinogen is less investigated, but could be of importance for development of severe AP. Interstitial activation of trypsinogen with enerokinase in experimental AP has changed the course of disease from mild to severe [55–57]. There is no strong evidence for intraductal activation of trypsinogen leading to AP [56].

Trypsinogen can be activated by enterokinase, trypsin and Cathepsin B [49,58]. Autoactivation of trypsinogen is shown in vitro, but is thought to be of less importance in vivo since the presence of Serine Protease Inhibitor Kazal type 1 (SPINK1), inhibits the process which is also regulated by pH (optimum around pH 5) and is increased in the presence of calcium [49,59]. Trypsin autolysis is

**Figure 1.** Activation of pancreatic proenzymes in acute pancreatitis. Trypsin is inhibited by several protease inhibitors. Resulting free trypsin activates important pancreatic proenzymes, yielding corresponding active enzymes and activation peptides.
Protease activation and inflammation in acute pancreatitis

Figure 2. Cellular events in a normal acinicell (left) and in AP according to the “co-localisation theory” (right). Protective mechanisms to inhibit premature protease activation are described to the left and how they are disturbed in AP to the right.

RER= Rough endoplasmatic reticulum, N= Nucleus, G= Golgi complex, L= Lysosomes, CV= Condensing vacuoles, ZG= Zymogen granulae, V= vacuole.

thought to be an important mechanism in the regulation of trypsin activity. This process is slowed down in the presence of calcium, allowing trypsin activity to persist much longer [49,60,61].

SPINK 1, also referred to as pancreatic secretory trypsin inhibitor (PSTI) is normally present in the pancreatic juice and is sufficient to inhibit trypsin activity if small amounts of trypsinogen is activated [49,62,63]. Another potent trypsin inhibitor is $\alpha_2$-macroglobulin ($\alpha_2$-M) which is present in blood, but due to its large size (725 kDa), it is not able to access the extra vascular space. A smaller (55 kDa) protein, $\alpha$-1PI, also called $\alpha$-1-antitrypsin, is present in plasma but has ability to diffuse into tissues. Half-life of the trypsin-$\alpha$-1PI complex in plasma is about 3.5 hours in vivo but trypsin is able to disso-
Trypsinogen and trypsin

There are two major forms of trypsinogen in pancreas, trypsinogen 1 (cathodal trypsinogen) and trypsinogen 2 (anodal trypsinogen). They are both around 25 kDa and the ratio between trypsinogen 1 and trypsinogen 2 is normally 0.8:1 in serum, but in AP levels of trypsinogen 2 increases more compared to trypsinogen 1, yielding a ratio of 1.3:1–2:1 [64–66]. Elevated levels of both trypsinogens, but to a higher extent trypsinogen 2, can discriminate patients with AP from other abdominal diseases. Elevated levels of trypsinogen 2 are also associated with severe AP [46].

Trypsin is a 24 kDa serine protease, able to cleave other proteins by binding to lysine and arginine residues inside the protein and hydrolyse their peptide bonds. Because of the potentially harmful effect of trypsin, protease inhibitors are present in pancreas and in blood. Since direct trypsin activity is difficult to measure, indirect signs of trypsinogen activation such as the complex of trypsin 2 coupled to α-1PI or TAP have been used in studies and high levels correlate with severity of AP [34,46].

The importance of trypsin in development of AP is further sustained by the finding of mutations in the trypsinogen gene, leading to increased risk of recurrent attacks of AP, often at young ages, for the patients [67,68]. A mutation in the gene for SPINK1 is more common in patients with AP compared to the population. Although the clinical implications for patients with this mutation are unclear, they are at higher risk of AP, probably because of a defect trypsin inhibitory effect of SPINK1 [68,69].

Carboxypeptidase B

Carboxypeptidase B is one of the major digestive enzymes [70]. It is synthesised as an inactive precursor of 45 kDa, procarboxypeptidase B (proCAP) in pancreatic acinar cells. Low levels of proCAP is found in human plasma from healthy individuals, but in patients with AP, proCAP leaks into the circulatory system and elevated levels discriminates these patients from patients with other abdominal disorders. When activated by trypsin, the active enzyme and activation peptide are formed in equimolar amounts [19,34].

Active carboxypeptidase B (aCAP) is a 35 kDa metalloprotease which has a proteolytic mechanism that differs from most other pancreatic proteases. It is an exoprotease, catalyzing the release of basic, carboxyl terminally (c-terminal) located aminoacids such as arginine, lysine, cysteine and ornithine [71]. This kind of proteolytic activity has been shown to affect the fibrinolytic, complement and bradykinin systems. No biological inhibitor to aCAP has been identified [62].

The activation peptide of procarboxypeptidase B (CAPAP), is the largest of the pancreatic activation peptides, 10 kDa, and can be measured in serum and urine from patients with AP. High levels of CAPAP in serum and urine, predicts development of severe AP if obtained within 72 hours from onset of symptoms [19,32,34,72].

Inflammatory changes in acute pancreatitis

Localized inflammation is the body’s initial physiological protective response to cell injury. Generally the process includes changes in the microvascular system such as vasodilatation, plasma leakage and leukocyte recruitment. Endothelial cells outlining the vessels are crucial in this process as they initiate the process leading to rolling, adherence and transendothelial migration of leukocytes into the tissue [73–75].
Protease activation and inflammation in acute pancreatitis

Leukocytes and adhesion molecules

Leukocytes include polymorphonuclear (PMN) granulocytes, monocytes/macrophages and lymphocytes. Activation of these cells leads to an increased tissue infiltration and an enhanced production of cytokines and other inflammatory mediators. Monocytes are produced in the bone marrow and circulate in the blood [73]. Upon migration into different tissues they undergo transformation into tissue macrophages. Activated mononuclear cells secrete proinflammatory chemokines such as tumour necrosis factor-α (TNF-α), Interleukin (IL) -1, IL-6 and IL-8. Monocyte activation is associated with severe AP and is seen early in the course of disease [76,77].

PMN granulocytes invade the pancreas early in the course of experimental pancreatitis, recruited by cytokines as well as factors of the complement system. They can synthesize and release both proinflammatory and anti-inflammatory cytokines. Release of proteolytic enzymes and formation of free radicals from activated granulocytes may contribute to aggravation of local destruction and systemic complications in AP [38,77–79].

The most abundant proteolytic enzyme in granulocytes is leukocyte elastase (PMN elastase), known for its ability to degrade different kind of tissues. It is released from PMN granulocytes early, especially in severe AP and is a marker of leukocyte activation [80]. Levels of leukocyte elastase is significantly more elevated in severe AP compared to mild disease during the first days from onset of symptoms [81–83].

Invasion of leukocytes into inflamed tissue includes rolling along the endothelium, adherence to the endothelium and transendothelial migration into the tissue [25,75]. Activation of endothelial cells leads to expression of the adhesion molecules platelet (P)- and endothelial (E)-selectins on the cell surface, thereby initiating rolling of leukocytes. Integrins are transmembrane glycoproteins expressed on neutrophiles regulating the adhesion of leukocytes to the endothelium. Upon activation they develop a high affinity for intercellular adhesion molecules (ICAMs) located on endothelial cells, and thereby the firm adhesion of leukocytes to the endothelium is mediated [25,75]. The transendothelial passage of leukocytes through the endothelium is believed to occur along the tight junctions between endothelial cells probably mediated by the platelet endothelial cell adhesion molecule-1 (PECAM-1), but an intercellular passage has also been shown [74,84]. Recruitment of leukocytes into pancreatic tissue is an important step in AP. The degree of activation of leukocytes correlates to the development of severe disease, and interference with leukocyte migration have reduced the severity of pancreatic and pulmonary damage in experimental AP [24,25].

The potent vasodilator platelet activating factor (PAF), is a phospholipid and a pro-inflammatory mediator which can cause increased capillary permeability and activation of leukocytes, mast cells and platelets [85]. It is released from cell membranes of endothelial cells, activated leukocytes and platelets. The production is closely tied to that of IL-1 and TNF-α so that inhibition of PAF attenuates IL-1 and TNF-α production and inhibition of either of these cytokines attenuates PAF production [76]. Several studies have shown decreased tissue damage and reduction of bacterial translocation in experimental AP when a PAF antagonist (lexipafant) is administered. Initial clinical trials with lexipafant treatment in severe AP have reported a significant reduction in the incidence of organ failure and also suppression of the inflammatory response [86,87]. However, the largest clinical double-blind, randomized, placebo controlled study failed to demonstrate a reduction in the frequency of new organ failure or mortality [88,89].
Cytokines and chemokines

Cytokines are low molecular weight proteins (usually 16–25 kDa) secreted by many different cell types in response to an injury or infection for recruitment of leukocytes. Proinflammatory cytokines also induce expression of selectins, increases capillary permeability, promotes extravasation of leukocytes and induce liver acute phase protein production [76, 77].

IL-1 and TNF-α are early proinflammatory cytokines, and primary inducers of IL-6 and IL-8. High levels have been found in pancreas in experimental AP and also their genes are expressed in pancreas after induction of AP [90]. Later, production in spleen, lung and liver is also seen [76]. Because of their short half-life in circulation and intermittent release, they are not always detectable in serum from patients with AP [16]. Increasing levels of IL-1 and TNF-α receptors correlates with severe AP in some reports [91, 92]. When investigating differences in cytokine polymorphism, no differences in TNF-α gene loci where seen between patients with AP and controls or between patients mild or severe AP [93].

IL-6 is a proinflammatory cytokine, (activator of lymphocytes) and the principle mediator of the acute phase response, thus regulating the production of CRP in the liver. IL-6 peaks after 24–48 hours and levels are more elevated in severe AP than in mild [76, 81, 92, 94].

IL-10 is an anti-inflammatory cytokine, down regulating a number of proinflammatory cytokines such as IL-1, TNF-α, IL-6 and IL-8 [95]. Pretreatment or treatment with IL-10 has reduced severity and delayed onset of pancreatic necrosis in experimental AP [16, 77, 95]. High levels of IL-10 are seen in patients with severe AP already within 24 hours from onset of symptoms, and levels stay elevated for the first week [16, 81, 92]. No difference in polymorphism frequencies of IL-10 gene loci has been found between patients with AP and controls or between patients with mild and severe AP [93].

IL-1 receptor antagonist (IL-1ra) is an anti-inflammatory cytokine, inhibiting the effect of IL-1. It is significantly more elevated in severe than in mild AP during the first 48 hours from onset of symptoms [92]. In experimental AP, administration of IL-1ra before or after the induction of AP has resulted in decreased pancreatic damage and decreased mortality [16, 96].

Chemokines

Chemokines (short for chemotactic cytokines) are small signalling molecules, roughly 70–130 amino acids with four conserved cysteines. Depending on their structural configuration they are classified as CXC respective CC chemokines. Their systematic names also include an L for ligand and the number of their respective receptor, but still they are mostly designated by their traditional names. Inflammatory chemokines are produced by many different tissue cells and by immigrating leukocytes in response to bacterial toxins and inflammatory cytokines. Their main function is to recruit leukocytes in infection and inflammation [77, 97].

IL-8 (CXCL8) is a chemotactic agent for neutrophils [76, 77] in which it causes degranulation and release of enzymes (especially leukocyte elastase) and toxic oxygen radicals. When overproduced this leads to tissue destruction. A genetical polymorphism increasing IL-8 production is more common in patients developing severe AP compared to controls (OR=2.07). No such differences where seen between controls and patients with mild AP [98]. Circulating levels of IL-8 are measurable in patients with AP within 24–48 hours from onset of symptoms and higher levels correlates to severe disease [76, 81].

Monocyte chemotactic protein 1 (MCP-1)
also named CCL2, is a potent chemotactant and activator of monocytes/macrophages and, to some extent, T cells, eosinophils and basophils. It is involved in acute as well as chronic inflammation and also in allergic reactions [97]. In experimental tissue transplantation MCP-1 is expressed in the immediate inflammatory response [97].

MCP-1 is induced in cerulein stimulated acinar cells, and it might be responsible for early attraction of monocytes/macrophages to the pancreas in AP [99]. In mice, MCP-1 inhibitors have been shown to inhibit severe experimental AP [100]. In patients with severe AP treated at the ICU, elevated MCP-1 levels has been reported to precede complications [101]. MCP-1 gene expression in humans is affected by an A/G polymorphism, with the G allele increasing MCP-1 production. Presence of the G allele (present in about 40% of controls) was associated with an odds ratio (OR) of 8.6 for developing severe disease (defined according to the Atlanta classification) in an earlier study, further indicating that MCP-1 is of importance for development of severe AP [102].

**Ischemia, microcirculation and microthrombosis**

Pancreatic blood flow is physiologically regulated by exocrine factors as somatostatin, secretin and cholecystokinin. A pancreatic lobule is supplied by a single vessel, often an end artery [103], which in most cases supplies islets first and then acini in a “insuloacinar” portal system [104]. This anatomy predisposes for the pancreatic susceptibility to ischemia seen in for example AP induced by low pancreatic blood perfusion due to systemic hypovolemia [105]. Also in an experimental setting prolonged ischemia induces AP, which has been verified histologically [106]. Ischemia is known to induce oxidative stress and especially in ischemia-reperfusion injury reactive oxygen species (ROS) seems to be involved [108]. If mediated by ROS or other factors, ischemia still seems to play a role in the development of severe AP and formation of pancreatic necrosis [107,108].

Induction of microthrombi by injection of microspheres as well as venous stasis leads to severe experimental AP [30,109], and ischemia and hypoperfusion converts mild experimental AP to severe [27,31]. The multiple intravascular thrombi seen in pathological tissue after severe necrotic pancreatitis [30,110–112] also supports this theory.

In vivo microscopy of rats have shown decreased tissue perfusion in the pancreatic head within an hour from onset of severe experimental AP, whereas it is unchanged or increased in mild disease [29,103,104,113]. These changes occur earlier than the central hemodynamic changes [29,108] and are probably effects of microvascular events. Impairment of the pancreatic microcirculation (seen as arterial constriction, lowered red blood cell velocity and microcirculatory stasis) occur with a marked interlobular variation in areas where necrosis later appears [29,103,104,107,113]. One possible explanation is that the microvascular changes predispose formation of microthrombi, which further increases local ischemia and leads to the formation of necrosis.

Inflammation may be a parallel or subsequent process to microvascular changes. Plaques of leukocytes in the vessels, leukocyte adherence to endothelium and leukocyte invasion are seen later than the above mentioned microvascular changes in experimental pancreatitis [114,115]. Increased permeability in pancreatic vessels is seen early, but without differences between mild and severe AP.

Histological findings in pancreas from animals with severe experimental AP as well as patients operated for severe necrotising AP has been compared to normal pancreatic tissue confirming microvascular changes in inflamed pancreas [111,115–117].
Oxygen derived free radicals

A free radical is an unstable and reactive metabolite because it contains an odd number of electrons in its outer orbital. Molecular oxygen is the major biological source of free radicals [118,119]. Under normal conditions most molecular oxygen undergoes controlled reduction in the mitochondria, forming water. A small proportion is only partially reduced, forming the cytotoxic free radicals, as a group called ROS. Normally, these are detoxified by endogenously produced intracellular scavengers [118,119].

Production of ROS increases when a period of intracellular anaerobic respiration is followed by re-oxygenation. If the rate of ROS production exceeds the capacity of the endogenous antioxidant mechanisms, oxidative stress develops [120]. Free radicals may then cause a chain reaction of lipid peroxidation leading to additional reactive intermediates. These are able of causing tissue injury such as damage of cell membranes and disruption of lysosomes. Antioxidants in plasma may be a protective factor for oxidative damage [121].

Different markers of oxidative stress have been measured in clinical and experimental AP with results indicating this process to be involved in the pathophysiology of the disease [121–124]. Profylactic treatment with antioxidant enzymes or allopurinol has reduced pancreatic damage in experimental AP, but treatment after onset of AP was less successful [118,119]. Recently a clinical randomized study could not show effect in incidence or severity of organ dysfunction for patients with severe AP treated with antioxidants [125].

Fibrinolysis

Thrombin makes fibrinogen polymerize resulting in bundles of fibrin and entrapped cells forming a blood clot. The fibrinolytic cascade starts with release of the tissue-type plasminogen activator (tPA) from endothelial cells initiating a limited conversion of plasminogen to plasmin [126–128]. In a first phase, a small amount of plasmin digests fibrin creating fibrin with C-terminal lysine residues. These residues generate a positive feedback in plasminogen activation and fibrinolysis in many ways. First, they are binding sites for both plasminogen activation and fibrinolytic effects of plasmin terminates in digestion of fibrin to soluble fibrin regeneration products (FDPs) [126–129] and D-dimer is one product in this family.

Thrombin activatable fibrinolysis inhibitor (TAFI)

TAFI is a plasma carboxypeptidase B which acts by the same type of proteolytic mechanism as aCAP, i.e. by cleaving c-terminal located lysine and arginine residues. It circulates in plasma in concentrations of 75 nM. Activation is mediated by thrombin and is enhanced by thrombomodulin. Activation by trypsin has also been shown [35,126,130].

The active form, TAFIa, is unstable in plasma, with an expected half life of ten minutes at body temperature [126]. In concentrations of 1.0–5.0 nM, TAFIa suppresses fibrinolysis by removal of the c-terminal lysine residues of partly destructed fibrin and thereby decreases the activation of plasminogen to plasmin. In higher concentrations (25–50 nM), a direct inhibitory effect on plasmin is seen [35]. It has been suggested that the same type of inhibition could be mediated by aCAP [126].
Fibrinolysis in acute pancreatitis

Systemic signs of consumptive coagulopathy and increased fibrinolysis are seen in the course of disease in severe AP [131,132]. Furthermore, in one study, elevated serum levels of D-dimers preceded signs of disseminated intravascular coagulation [133], indicating an earlier coagulative-fibrinolytic process going on. High levels of D-dimers at admission have also been reported to predict development of severe AP [134].

Necrosis formation

The pathogenetic mechanisms leading to formation of pancreatic necrosis are not fully understood. Histological studies show a faster destruction of acinar cells compared to the interlobar and/or extrapancreatic fat cell necrosis which can be seen for several weeks [36,37]. Proteolytic and lipolytic enzymes might contribute to this rapid degradation of the acinar cells [49,51]. In pancreas from patients undergoing surgery within two days after onset of fulminant AP, viable pancreatic tissue were seen even when contrast enhanced CT had shown low enhancement and no perfusion [135]. Studies in experimental AP also reports impaired circulation before development of necrosis, indicating that impaired perfusion contributes to formation of pancreatic tissue necrosis [29,103,104,113]. The necrotic destruction of the cell generates a profound inflammatory reaction. Apoptosis (regulated cell death), on the other hand, is a controlled process ending in digestion of the apoptotic bodies by macrophages without inflammatory activation. In mild AP there is a high apoptotic activity which is replaced by a high necrotic activity in severe AP. This may be a result of the trauma to the acinar cell, but may also be a result of stimuli from factors changing apoptosis to necrosis such as reactive oxygen species [136].

Prediction of severity

The ability to differentiate severe from mild AP in the clinical situation has been evaluated in several trials, resulting in a sensitivity of approximately 40% for an experienced clinician to predict severity on admission. This means that the majority of patients who develops severe AP are initially misidentified as mild [17,18]. Since strict observation and prompt treatment of systemic complications is assumed to lead to better outcome concerning mortality and morbidity for patients with severe disease, much effort has been put into the issue of finding a good marker or a scoring system for prediction of severe AP at admission to the hospital [14,16].

For treatment studies and research concerning pathophysiology of AP, early prediction of severity is crucial for identification and inclusion of only severe cases in the study. If patients with mild as well as severe AP are included in treatment studies, results will be delicate to interpret and the studies will need to be very large, because of the high percentage of patients with mild disease that will not benefit from the treatment but diluting the results. There are also ethical aspects of testing new treatments in a group of patients if a high percentage will not benefit, but may risk side effects and complications from the treatment.

Today most clinical studies use the Atlanta Classification for retrospective evaluation of mild and severe AP [4]. According to this consensus, severe AP is classified as AP with systemic or local complications. Systemic complications include circulatory, respiratory and kidney dysfunction as well as signs of coagulopathy or other dysfunctions after the initial dehydration is treated. Local complications include pancreatic necrosis more than 30%, pancreatic abscesses and pseudocysts [4].
Clinicobiochemical scoring systems

Clinicobiochemical scoring systems are not usually used at admission in clinical practice as they are complex and time consuming. They might be used before including patients in studies and for evaluation of patients with severe AP, especially in the ICU.

The Ranson criteria include evaluation of eleven clinical and biochemical parameters on admission and during the initial 48-hour period. Three or more positive signs at admission indicate severe disease [137,138]. The predictive power of Ranson criteria at admission is poor and at 48 hours the sensitivity and specificity reported is approximately 45% and 98% respectively [139,140].

The Acute Physiological and Chronic Health Evaluation II (APACHE II) system can be used at any time during the course of disease. It is based on 12 physiologic variables, patient age and chronic health status [141]. In some studies, it is reported to predict severe AP at admission somewhat better than Ranson criteria, with a sensitivity and specificity of around 70% [139,140,142], but it is more often used for evaluation of disease severity and progress in the ICU.

Biochemical markers of severity

The ideal marker for prediction of severity in AP should be a quick, harmless and clinically available blood or urine test, specific for AP. It should be possible to use as a diagnostic tool and have a high sensitivity as well as a high specificity for prediction of severe AP. So far, no marker fulfilling these criteria has been identified.

Pancreatic enzymes are often elevated in AP because they leak into the circulatory system. Most proenzymes as well as amylase and lipase do not discriminate between mild and severe disease [19,39,64]. Levels of serum and urinary trypsinogen 2 are elevated in AP, and to a higher extent in severe disease. The sensitivity and specificity for prediction of severe AP for different cut offs and in different studies ranges from 26 to 90% and from 77 to 96% respectively [40,41] for samples obtained within 12–24 hours from admission to the hospital. Urinary trypsinogen 2 is available as a dip-stick, but it seems to be better for the diagnosis of AP than for predicting severity of disease [143].

Activation peptides such as TAP and especially CAPAP are reported to predict severe disease more accurate. For urinary CAPAP, a sensitivity of 81–92% and specificity of 83–89% is reported for samples obtained within 48–72 hours from onset of symptoms [19,34]. However, the analytic methods for activation peptides are not available for routine use today. Elevated levels of the complex trypsin-α-1PI discriminate mild from severe AP with a sensitivity of 70–95% and a specificity of 64–72% [40,41], but is not available for clinical use.

Markers of inflammation are not specific for AP, but can be used to discriminate between mild and severe disease. CRP is the most used test in clinical practice today, even though it peaks 48–72 hours after onset of symptom. It shows low predictive value for severe AP the first 48 hours after onset and hence, is not useful as a predictor of severe AP [19,20].

The proinflammatory interleukins IL-1 and TNF-α correlates with severity in some reports, but are difficult to measure because of their short half life in blood and the intermittent release of TNF-α [13,144]. IL-6 has a well documented ability to predict severe disease at an early stage (peak values approximately 24–48 hours from onset of symptoms) with a sensitivity of 89–100% and a specificity of 86–67% [13,81,83,95,145] and is available for clinical use in some centers. IL-8 has shown a lower sensitivity in some studies, but its timecourse and peak parallels IL-6 [13,81,92]. Leukocyte elastase is released by PMN leukocytes early in the course of AP
and has been reported to have a sensitivity of 67–85% and a specificity around 62–89% for prediction of severe disease [80,81,144,146]. The anti-inflammatory IL 1-ra is reported to distinguish between mild and severe AP with a sensitivity of 82% and a specificity of 86% for samples obtained within 48 hours from onset of symptoms [92] and the figures for IL-10 reported is similar [81]. Both IL-6 and leukocyte elastase are available as automated assays, even though they are not in routine use in most centers.

Adhesion molecules such as different selectins and the soluble form of ICAM-1 have shown different results in the few studies done and does not seem to be usable as admission markers for severe AP [80,147].

Procalcitonin (PTC) is the precursor of calcitonin. Its site of production and its pathophysiological role is unknown, but it is a rapid general acute-phase reactant not specific for AP. Levels of PTC correlates with severity of AP within 24 hours from admission. A semi-quantitative test strip for serum is available and has shown a sensitivity of 71% and a specificity of 84% at admission [139]. Procalcitonin may be useful for prediction of severe AP in the future.

**Radiological findings**

Contrast enhanced CT is widely used for diagnosing pancreatic injury and evaluating surrounding tissues [148], even though the interobserver variance among different radiologists is reported to be high [149,150]. Extended pancreatic necrosis is detectable on CT, but minor necrotic areas are missed in 50% of cases, and pancreatic necrosis may not develop in the first 48 hours. Moreover, the presence and amount of pancreatic necrosis does not strongly correlate with organ failure [14]. Finding of certain pancreatic abnormalities have been graded by the so called Baltazar score, and patients with a higher score are more likely to develop severe disease even though the highest diagnostic and predictive accuracy is seen after the first week of disease [14,148]. The risk of adverse effects of contrast infusion has been studied with conflicting results. An increase in severity and mortality has been reported after contrast infusion in experimental pancreatitis [13,135].
Aims

• To develop a method for measurement of aCAP in AP.

• To compare markers of protease activation and inflammation as early predictors for severity in AP.

• To study the early time course of markers of pancreatic leakage, protease activation and inflammation in AP, and how their levels differs between mild and severe AP.

• To investigate if aCAP inhibits fibrinolysis

Material, methods and results

Materials and methods, Paper I

The aim of paper I was to develop an assay for measurement of aCAP in AP. An ELISA was established, using two antibodies against pure aCAP. Levels of aCAP in patients with AP and healthy controls were compared.

Purification of aCAP

Pancreatic juice was collected from patients who had their main pancreatic duct drained after major pancreatic surgery [32]. A 20 ml sample of the juice was applied to an affinity chromatography column (2.0 cm x 20 cm) with aprotinin (Bayer Sverige AB, Sweden) coupled to the gel for separation of trypsinogen and trypsin [64].

To identify fractions containing proCAP, trypsin (bovine pancreatic trypsin type 1; Sigma, St.Louis, MO., USA) was added (40 µl 0.05 mg/ml to a final concentration of 0.01mg/ml) to a 160 µl sample of respective fraction, before incubation at room temperature for 1 hour for activation. The aCAP activity was tested, using the chromogenic substrate hippuryl-arginine (N-Benzoyl-Gly-Arg, SIGMA chemical company, St.Louis, MO., USA) [151] measuring the cleavage of arginin. The proteins were also visualised by agarose gel electrophoresis at pH 8.6 [152]. Fractions containing proCAP were transferred to a 0.02 M Tris buffer (pH 7.5) using a PD-10 column and then applied to an ion change chromatography column as previously described [32]. For identification of fractions containing proCAP, aCAP activity after activation was tested as above and agarose gel electrophoresis showed a single slightly anionic band [32].

Fractions containing pure proCAP (2 x 0.5 ml) were transferred to a 0.02 M Tris buffer (pH 8.2) using a PD-10 column (Pharmacia, Uppsala, Sweden). For activation, 0.05 mg/ml trypsin (bovine pancreatic trypsin type 1; Sigma, St.Louis, MO., USA) was added to a final concentration of 0.01 mg/ml and a final volume of 1.26 ml and incubated at room temperature for 1 hour. After activation, 3.5 ml of the sample was applied to an ion change chromatography column (Mono Q, Pharmacia Biotech, Uppsala, Sweden) equilibrated with 0.02 M Tris, pH 8.2 buffer and eluted with a linear gradient of NaCl from 0 to 0.35 M at 1 ml/min. Fractions were analysed for aCAP activity with the chromogenic substrate hippuryl-arginine [151].

Fractions containing aCAP showed a single cationic band on agarose gel electrophoresis (agarose 1% w/vol Sigma, St.Louis, MO.,USA, buffer: 60 mM barbital sodium, 10 mM 5.5-dietylbarbituricacid, 2 mM calciumlactate, pH 8.6, 220V). The molecular mass was determined using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis with sample and running buffer, reducing agent and standard used according to instructions from the manufacturer (NuPAGE...
Protease activation and inflammation in acute pancreatitis

Bis-Tris Gels, 4–12%, NuPAGE MES buff kit, NuPAGE reducing agent, Multi Marle standard, Invitrogen, Carlsbad, CA;USA) and found to be around 30–35 kDa. Protein mass was determined by the method of Lowry [153]. Pure aCAP was concentrated by ultrafiltration for 60 minutes (4°C) on a Centricon filter (membrane NMWL 10 000, Amicon Inc, Beverly, MA, USA).

Antibodies
Rabbits were immunized with subcutaneous injections of 0.1 mg aCAP mixed with Freund’s complete adjuvant (SIGMA chemical company, St.Louis, MO., USA), repeated every third week with 0.1 mg aCAP mixed with Freund’s incomplete adjuvant (SIGMA chemical company, St.Louis, MO., USA). Blood samples were drawn after 9, 11 and 13 weeks, incubated in 4°C overnight and centrifuged (g = 1600) for 10 minutes for separation of serum. The antibody titers and specificities were tested by immune electrophoresis using a 1% (w/vol) agarose gel (SIGMA chemical company, St.Louis, MO., USA) and buffer (60 mM barbital sodium, 10 mM 5.5-dietylbarbituricacid, 2 mM calciumlactate, pH 8.6). Pancreatic juice or pure aCAP was applied in wells in the gel and electrophoresis run at 220V. Different antisera were added in the slits between the wells before the gel was incubated in a humid chamber at room temperature over night to allow precipitation of immune complexes. After washing with 0.15 nmol NaCl for 24 hours, the gel was dried at 65°C for 15 minutes. Staining with 0.3% Coommassie (Coomassie brilliant blue R-250, BDH Laboratory, England) diluted in a solution of 5.95 mol/l ethanol and 1.75 mol/l acetic acid was performed before washing in the 5.95mol/l ethanol and 1.75 mol/l acetic acid solutions. Two rabbits developed sufficient titers of immunoreactivity, but a cross reaction with proCAP was seen on the immune electrophoresis.

Immunoglobulin fractions of each antiserum were obtained using a 0.9 x10 cm protein A column (Protein A Sepharose CL-4B, Amersham Pharmacia Biotech, Uppsala, Sweden) prepared according to instructions from the provider. A sample of 5 ml antisera and 0.5 ml 1M Tris pH 8 was applied to the column before running with 0.1 M Tris, pH 8 for 2 hours and 0.01 M Tris, pH 8 for 2 hours, both at 20 ml/h. The immunoglobulin was eluted using 0.1 M Glycin, pH 3 (2 ml/fraction) into tubes containing 50µl 1 M Tris, pH 8. The antibody concentration was measured with spectrophotometry.

ELISA procedures
The antiserum was diluted in buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃, 0.02% (w/v) azid, pH 9.6) to a concentration of 10 mg/l and applied (100 µl/well) to a microtiter-plate (Maxi Sorp, Fisher Scientific, Göteborg, Sweden) before incubation overnight at room temperature. After washing with buffer (0.1 M Tris, 0.05 M CaCl₂, pH 7.4) the wells were blocked with 150 µl of 1% (w/v) bovine serum albumine (BSA, SIGMA chemical company, St.Louis, MO., USA, in buffer 0.1 M Tris, 0.05 M CaCl₂, pH 7.4, 4 hours) and stored at 4°C until use. Each well contained 100 µl 0.2% (w/v) BSA in buffer (0.1 M Tris, 0.05 M CaCl₂, pH 7.4) during storage.

An amount of 700 µl of the second antiserum was diluted in 300 µl 0.1 M NaHCO₃ to a final concentration of 5 mg/ml. The mixture was dialysed (Spectra Por MWC 6–8000, Spectrum Medical Industries. Inc. Houston, TX., USA) at room temperature in 1 l of 1 M NaHCO₃ over night. An amount of 120 µl biotin (N-Hydroxysuccinimidobiotin, SIGMA chemical company, St.Louis, MO., USA) 1 mg/ml diluted in dimetylsulfoxid (DMSO, SIGMA chemical company, St.Louis, MO., USA) was added and incubated for 4 hours at room temperature before dialysis against 0.01 M phosphate buffered saline (PBS, SIG-
MA chemical company, St. Louis, MO., USA, containing 0.01 M Na₂HPO₄, 0.15 M NaCl, 0.1% azid), pH 7.2, at 4°C overnight. The biotinylated antibodies were stored at 4°C and diluted to 1/1000 (in buffer 0.1 M Tris, 0.05 M CaCl₂, pH 7.4) before used.

For dilution of standard, samples and avidin conjugate, a buffer 0.2% (w/v) BSA, 0.1 M Tris, 0.05 M CaCl₂, pH 7.4, was used and for washing the same buffer without BSA. Purified aCAP in concentrations from 0.078–1.25 nmol/l (2.5–40 µg/l) was used as standard. A serum pool containing aCAP at a concentration of 0.6 nmol/l was used as a control. The standard was stored at 4°C and controls at −20°C. Coated microtiters plates were washed and 10 µl of samples (diluted at least 1/5) or standards were applied to the wells before incubation for 2 hours at room temperature. After washing, 100 µl of the second antibody was applied to each well and incubated for 1 hour at room temperature. After further washing, 100 µl avidin-conjugated alkaline phosphatase (Biorad, CA.,USA) diluted 1/1000 was applied and incubated for 1.5 hours. Phosphatase substrate (5 mg tablets, SIGMA chemical company, St. Louis, MO., USA) was diluted in 10 ml 1.0 M diethanolamine 1 mM MgCl₂ buffer, pH 9.8, and 100 µl was applied to each well. Absorbance was measured at 405 nm.

Analysis of proCAP and CAPAP

Immunoreactive (ir) proCAP and CAPAP were analysed using ELISA methods described previously [19].

Gel filtration

Gel filtration was performed on a 0.9 x 60 cm G75 column (Sephadex Superfine G75, Amersham Pharmacia Biotech, Uppsala, Sweden) with buffer 0.2% (w/v) BSA, 0.1 M Tris, 0.05 M CaCl₂, pH 7.4 used for elution at a flow rate of 3 ml/h. The application volume was 300 µl (serum or dilutions of aCAP or proCAP) and the sample volume was 600 µl.

Characterization of pure proCAP and aCAP

Pure proCAP (10 µl, 100 nmol) or pure aCAP (50 µl, 100 nmol) was mixed with buffer (0.2% (w/v) BSA, 0.1 M Tris, 0.05 M CaCl₂, pH 7.4), to a final volume of 1500 µl. 300 µl of respective mixture was subjected to gel filtrations and fractions were then assayed in the aCAP ELISA.

Interactions between aCAP and serum proteins in vitro

Pure aCAP (10 µl, 10 nmol), was mixed with 300 µl fresh human serum (diluted 1:1 in buffer 0.1 M Tris, 0.05 M CaCl₂, pH 7.4) or with 300 µl buffer 0.2% (w/v) BSA, 0.1 M Tris, 0.05 M CaCl₂, pH 7.4, and incubated for 24 hours at room temperature. The samples were then subjected to gel filtration as described above, and elution fractions were assayed in the aCAP ELISA for quantification of ir-aCAP.

Patients and blood samples

Blood and urine samples were obtained within 72 hours from onset of symptoms from 25 patients with acute pancreatitis admitted to Malmö University Hospital. Acute pancreatitis was defined as acute abdominal pain and an increase in serum amylase level at least three times the upper reference limit (i.e 3 x 0.8=2.4 µkat/l). Severity was classified according to Atlanta classification by reviewing the clinical history of the patients [4]. Maximum CRP levels for the patients were obtained from the review of the clinical history. Blood samples were obtained from 10 healthy volunteers: 5 males and 5 females aged 25 to 54 years. The blood sample was allowed to clot and then centrifuged at 4°C (g=1600)
Protease activation and inflammation in acute pancreatitis

within 6 hours. The serum was stored at –20°C. Urine samples were stored at –20°C. The procedure was approved by the Research Ethics Committee of Lund University (LU-47-00). All patient samples were first assayed in three different dilutions (1:25, 1:50 and 1:100) and if necessary in higher or lower concentrations depending on the amount of ir-aCAP. A dilution of at least 1:5 was used for all samples.

Statistical analysis
Data analysis was performed using Macintosh Stat View software. Fisher’s r to z test was used to study correlations.

Results, Paper I
acAP assay and cross reaction with proCAP
A standard dilution curve for ir-acAP and the cross reaction with pure proCAP is shown in Figure 3. The sensitivity of the assay is around 0.05–0.1 nmol/l (1-2 µg/l) and the cross reaction with proCAP is less than 10% compared to ir-acAP. The inter-assay coefficient of variation (CV) was 1.7% between 0.078 and 1.25 nmol/l. All samples were diluted and analysed in this interval.

Normal serum
Serum from healthy subjects contained ir-acAP in concentrations ranging from 0.17–0.33 nmol/l (mean 0.24, SD 0.06 nmol/l). Since a dilution of 1:5 is the lowest possible in the ELISA, concentrations are too low to characterize the measured immunoreactivity by dilution curves or to study recovery after gel filtration.

Patient data
Median age of the 25 patients (15 males, 10 females) was 48 years (range 26–92). The probable ethiology was alcohol in 9 patients, gallstone in 8, unknown in 2 and other known causes in 5 cases. There were 20 patients with mild and 5 patients with severe AP according to Atlanta classification. Two patients developed pancreatic necrosis and one underwent surgery. One patient died. The median value for maximum CRP levels of the patients was 182 mg/l (range 12–519).

Figure 3. The absorbance of a standard dilution curve of acAP and proacAP respectively, measured in the acAP assay showing the cross reaction with proacAP.
ir-aCAP in patient serum
Serum samples from patients with AP contained ir-aCAP in concentrations from 0.6 to 158 nmol/l. All samples with values in the lower half of the standard curve had dilution curves parallel to the standard curve. Five samples with high values had dilution curves that to some extent diverged from the slope of the standard curve; this could be overcome by diluting the samples more.

Characterization of pure proCAP and aCAP by gel filtration
The distribution of immunoreactivity in the aCAP assay after gel filtration of pure proCAP and pure aCAP is shown in Figure 4. Pure aCAP was eluted in a larger volume due to its smaller molecular size. Serum samples from patients with AP mainly showed two different patterns depending on the amount of aCAP in serum. The patterns from a patient with low and a patient with high serum values are shown in Figure 5. Small amounts of aCAP correspond to a small peak at the molecular size of proCAP. High levels of aCAP showed two peaks, one in the volume expected for proCAP and one in the same volume as aCAP.

Ir-aCAP in relation to s-proCAP, u-CAPAP and maximum CRP levels
For patient samples, the level of ir-aCAP in serum was plotted against the level of ir-proCAP in serum and ir-CAPAP in urine of the same patient taken at the same time. The correlation between levels of ir-aCAP and ir-proCAP was not statistically significant (r=0.24, p=0.373) but the correlation between levels of ir-aCAP and ir-CAPAP showed a statistically significant correlation (r=0.40, p=0.045). There was no statistically significant correlation between the levels of ir-aCAP and maximum CRP levels (r=0.072, p=0.742).

Recovery of ir-aCAP after gel filtration
The levels of ir-aCAP in 9 serum samples from patients with AP were measured directly in the aCAP assay at a dilution of 1:50 and compared to the amounts of ir-aCAP in the same sample estimated as the area under the true aCAP curve (peak) obtained after gel filtration (AUC). The correlation was statistically significant (r=0.913, p=0.0002), although the levels estimated after gel filtration for all samples were only about 20–25% of the levels measured directly.
Interaction between aCAP and serum proteins

When aCAP was incubated with serum or buffer for 24 hours, no immunoreactivity was unaccounted for measured in the aCAP assay. After gel filtration, all immunoreactivity was eluted corresponding to free aCAP.

Materials and methods, Paper II and Paper III

In paper II, markers of protease activation (aCAP and CAPAP) and of inflammation (MCP-1 and CRP) at admission were evaluated as predictors of severe AP. The aim of paper III was to compare markers of different early pathophysiological events; i.e protease activation (aCAP, CAPAP), pancreatic leakage (proCAP, trypsinogen 2) and inflammation (MCP-1, CRP) over time in patients with mild and severe AP.

Patients

In all, 140 patients (65 men, 75 women), admitted to Malmö University Hospital with 156 events of AP between March 2000 and September 2005 were included in the studies. Patients (18 years and older) with AP and samples obtained within 72 hours from onset of symptoms, were eligible for inclusion. In the analysis, only the first event for each patient was included. Patients who had at least one sample obtained on all of the first three consecutive days from onset of symptoms were eligible for inclusion in the time course analysis in paper III. If a value during these days was missing, the patient was excluded from the time course analysis regarding that specific marker resulting in different number of patients for different markers (n=44–61 except for the amylase analysis where n=27).

Informed written consent was obtained from all patients before entering the study. The studies were approved by the Ethics Committee at Lund University (LU 47-00).

The definition of AP and different etiologies is described in paper II and III. Of the 140 cases, 16 patients (11 percent) were classified as severe AP according to the Atlanta classification [4].

Samples and assays

Serum and urine samples were collected immediately following a patient’s inclusion in the study and on the following three days.

Serum levels of aCAP were determined by the assay described in paper I. Levels of trypsinogen 2 and proCAP in serum and CAPAP in serum and urine were determined us-
ing double-antibody ELISA methods as previously described [19,64]. MCP-1 levels in serum and urine were also analysed with an ELISA method (Quantikine, R&D Systems, Minneapolis, USA.

Analysis of CRP, Hb and pancreatic amylase samples were not part of the original study protocol. These parameters were analysed by the Department of Clinical Chemistry, Malmö University Hospital [154] and results were retrieved from clinical notes.

Statistical methods

SPSS 15.0 (SPSS Inc., Chicago, USA) was used for all statistical analyses. The Mann–Whitney U test was used to test differences in the biochemical markers between patients with severe and those with mild AP. Differences were considered statistically significant when the p-value was less than 0.05. In paper II, receiver operating characteristics (ROC) curves in relation to prediction of developing severe AP were plotted and areas under the curves (AUC) were calculated. Cut-off values for CAPAP, aCAP and MCP-1 were derived from the ROC curves based on optimal sensitivity and specificity, and high positive and low negative likelihood ratios, which were calculated for different cut-off levels. Values above the cut-off were investigated as predictors of developing severe AP using unconditional logistic regression yielding odds ratios (OR) with 95% confidence intervals. All analyses were subsequently adjusted for age, sex, and aetiology. Moreover, all analyses were repeated stratified for aetiology (biliary, alcohol, or other/unknown). High values were combined to create a score from 0 (no high levels) to 3 (high levels for MCP-1, CAPAP, and aCAP) for each subject. Corresponding ORs were calculated as above. In paper III, The Wilcoxon test was used to test differences in concentration of markers for Day 2 versus Day 1 and for Day 3 versus Day 2. Differences in median values between the severe and the mild group for each marker on Days 1 to 3 in patients selected for the time course analysis were compared to corresponding differences in the analysis including all patients.

According to the computerized records at Malmö University Hospital, 793 events of AP were registered in Malmö during the study period. Among these 793 events, 128 of the 140 patients included in the present study were found. All events in other patients (n=665) were compared to included patients in the present study (n=140) with regard to gender distribution and median age.

Results, Paper II and III

Patient characteristics

The median age (58.1 years, range 20 to 96 years), and gender distribution (54% females/46% males) for included patients (Table 1), was similar to other patients diagnosed with AP during the study period (median age, 61.2 years; 51% females). Patients with severe AP (n=16, 11%), were older than those with mild disease. Both alcohol and gallstones as the most probable aetiological factor were more common in severe than in mild disease, Table 1. The median time from symptom onset until inclusion in the study was 24 hours for patients with mild as well as with severe AP. The group selected for time course analysis was slightly older compared to all included patients, but the groups were similar regarding gender distribution and aetiology. Patient characteristics are shown in Table 1.

Markers

Differences between mild and severe cases, Paper II

Levels of CAPAP in serum and urine, aCAP in serum and MCP-1 in serum and urine at admission (4–72 hours from onset of symptoms, median 24 hours), were all significantly
Protease activation and inflammation in acute pancreatitis

Table 1. Age, sex and aetiology in patients with mild and severe acute pancreatitis (AP).
Among all patients, and among patients with information on day 1, 2, and 3 from onset of symptoms.

<table>
<thead>
<tr>
<th>Factor</th>
<th>All patients</th>
<th>Patients with samples on day 1, 2 and 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mild AP (n=124)</td>
<td>Severe AP (n=16)</td>
</tr>
<tr>
<td>Age mean (SD)</td>
<td>57.4 (18.2)</td>
<td>63.6 (14.9)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>58 (46.8)</td>
<td>7 (43.8)</td>
</tr>
<tr>
<td>female</td>
<td>66 (53.2)</td>
<td>9 (56.3)</td>
</tr>
<tr>
<td>Aetiology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>biliary</td>
<td>59 (47.6)</td>
<td>7 (43.8)</td>
</tr>
<tr>
<td>alcohol</td>
<td>26 (21.0)</td>
<td>5 (31.3)</td>
</tr>
<tr>
<td>unknown</td>
<td>32 (25.8)</td>
<td>3 (18.8)</td>
</tr>
<tr>
<td>other</td>
<td>7 (5.6)</td>
<td>1 (6.3)</td>
</tr>
<tr>
<td>CRP mean (SD)</td>
<td>133 (100)</td>
<td>275 (65)</td>
</tr>
</tbody>
</table>

* Mean and standard deviations in italics.

Table 2. Median and 2.5 percentile to 97.5 percentile values for the biochemical markers for patients with mild and severe acute pancreatitis.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Mild</th>
<th>Severe</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>s-CAPAP (nmol/l)</td>
<td>0.95 (0.0–27.9)</td>
<td>5.05 (0.6–27.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>u-CAPAP (nmol/l)</td>
<td>2.25 (0.0–375.2)</td>
<td>60.25 (0.0–479.4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>aCAP (nmol/l)</td>
<td>11.35 (0.43–92.3)</td>
<td>20.5 (3.7–81.4)</td>
<td>0.01</td>
</tr>
<tr>
<td>s-MCP-1 (μg/l)</td>
<td>222.5 (97.0–1120)</td>
<td>1094 (126–237,000)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>u-MCP-1 (μg/l)</td>
<td>535 (30.0–9231)</td>
<td>3611 (500–45,600)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CRP max (mg/l)</td>
<td>122 (9.0–379)</td>
<td>266 (180–430)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

higher in patients developing severe AP than in patients with mild disease as described in Table 2. Results for maximum CRP values are shown for comparison since this is an analysis often used by clinicians. CRP (information on 137 patients) and haemoglobin-count (136 patients) at admission had an area under the ROC curve of 63% and 58% respectively.

The AUC for MCP-1 in serum was larger compared to the other markers in both serum and urine, shown in Figure 6. Sensitivity, spe-
Figure 6. Receiver operating characteristics (ROC) curve of serum (a) and urine (b) markers. Area under the curve (AUC) and $p$-value for the AUC (using AUC=50% as a reference) in brackets.
specificity and likelihood ratios were calculated for different cut-off levels, and MCP-1 in serum showed the highest values, described in Table 3. Calculating these estimates separately for younger patients and patients older than 70 years showed similar cut-off levels (results not shown).

MCP-1 in serum at admission showed the highest OR associated with severe AP; adjustment for age and sex did not change these estimates as shown in Table 4. ORs were even higher in cases related to gallstone disease, but the number of patients was small and confidence intervals wide (results not shown). The analysis combining several markers as a score from 0 to 3 did not show any ORs higher than those seen for MCP-1 alone (results not shown).

Differences between mild and severe cases, Paper III
In the first analysis in paper III, markers for all patients where compared between mild and severe AP for day 1, 2 and 3 from onset of symptoms. CAPAP levels were significantly higher in severe AP as compared to the mild group on all days, with the largest differences on Day 2. The concentration of aCAP did not differ significantly between patients with mild and severe AP. Trypsinogen 2 showed significantly higher levels in the severe AP group on all three days, whereas levels of proCAP and amylase did not differ significantly between patients with mild and severe AP. MCP-1 was significantly higher in severe AP during the first three days, whereas CRP showed statistically significant differences between the groups on Day 2 and Day 3.
Time course analysis
In the time course analysis levels of markers each of the first three days in the same patients were analysed. Median levels for the markers over time are shown in Figure 7, 8 and 9. Levels of CAPAP in serum were high in severe AP during the first 48 hours, with a significant decline between Day 2 and Day 3, whereas CAPAP levels in urine peaked on the second day. Levels of aCAP were higher in the mild group on Day 2. All markers of leakage (pro-CAP, trypsinogen 2, and amylase) showed the highest values on Day 1, declining over time as seen in Figure 8. MCP-1 serum levels in severe AP were high during the first 48 hours with a significant decline between Day 2 and Day 3, and MCP-1 levels in urine peaked on the first day. CRP values were low on Day 1 but significantly higher on Day 2. The pattern of differences between mild and severe cases on Days 1 to 3 was similar for all markers, except amylase, when patients included in the time course analysis were compared to the whole study population (data not shown).

Materials and methods, Paper IV
In this paper, the capability of aCAP to inhibit fibrinolysis was studied. Two different models for measurement of fibrinolysis was developed and used in the study.

Table 4. Risk of severe and mild acute pancreatitis (AP) associated with different biochemical markers. Odds ratios (OR) with 95% confidence intervals in brackets.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Mild AP</th>
<th>Severe AP</th>
<th>OR</th>
<th>OR*</th>
<th>OR**</th>
</tr>
</thead>
<tbody>
<tr>
<td>s-CAPAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2nmol/l</td>
<td>79</td>
<td>4</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>≥2nmol/l</td>
<td>44</td>
<td>12</td>
<td>5.4 (1.6–17.7)</td>
<td>5.3 (1.6–17.7)</td>
<td>5.5 (1.6–18.4)</td>
</tr>
<tr>
<td>u-CAPAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20nmol/l</td>
<td>92</td>
<td>6</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>≥20nmol/l</td>
<td>32</td>
<td>10</td>
<td>4.8 (1.6–14.2)</td>
<td>5.7 (1.7–18.3)</td>
<td>4.8 (1.5–14.9)</td>
</tr>
<tr>
<td>aCAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;15nmol/l</td>
<td>77</td>
<td>5</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>≥15nmol/l</td>
<td>46</td>
<td>11</td>
<td>3.7 (1.2–11.3)</td>
<td>3.8 (1.2–11.9)</td>
<td>3.7 (1.2–11.3)</td>
</tr>
<tr>
<td>s-MCP-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;500</td>
<td>105</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>≥500</td>
<td>18</td>
<td>14</td>
<td>40.8 (8.5–19.5)</td>
<td>53.0 (9.7–289)</td>
<td>78.6 (9.6–643)</td>
</tr>
<tr>
<td>u-MCP-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1000</td>
<td>88</td>
<td>4</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>≥1000</td>
<td>36</td>
<td>12</td>
<td>7.3 (2.2–24.3)</td>
<td>7.0 (2.1–23.5)</td>
<td>8.1 (2.3–28.3)</td>
</tr>
<tr>
<td>CRP max</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;200</td>
<td>90</td>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>≥200</td>
<td>34</td>
<td>15</td>
<td>39.7 (5.0–312)</td>
<td>41.9 (5.2–337)</td>
<td>41.8 (5.2–335)</td>
</tr>
</tbody>
</table>

OR* adjusted for age and sex  OR** adjusted for aetiology
Figure 7. Time course analysis for markers of activation; serum CAPAP (a), urine CAPAP (b) and serum amylase (c). Statistically significant differences ($p < 0.05$) between levels on day 2 vs. day 1, and between day 3 vs. day 2, noted as */*.

Figure 8. Time course analysis for markers of leakage; serum proCAP (a), serum trypsinogen 2 (b) and serum amylase (c). Statistically significant differences ($p < 0.05$) between levels on day 2 vs. day 1, and between day 3 vs. day 2, noted as */*.
Figure 9. Time course analysis for markers of inflammation; serum-MCP-1 (a), urine MCP-1 (b) and serum CRP (c), median values. Statistically significant differences (p<0.05) between levels on day 2 vs. day 1, and between day 3 vs. day 2, noted as /*/.

aCAP

Purification of aCAP was performed as described for paper I. Purified aCAP was kept frozen (−20°C) and the activity was tested with a chromogenic substrate hippurylarginine (N-Benzoyl-Gly-Arg, SIGMA chemical company, St.Louis, MO., USA) [151] before use. Absence of trypsin was tested with the chromogenic substrate Nα-benzoyl-DL-arginine-p-nitroanilide (BAPNA) hydrochloride (SIGMA chemical company, St.Louis, MO., USA) [155].

Fibrinolysis model:
D-dimer measurements

The fibrinolytic activity in human plasma was studied by measuring levels of D-dimers. First, different dilutions of plasma were tested, and a dilution characterized by increasing fibrinolysis over time (indirectly measured as increasing levels of D-dimers) was chosen. This dilution was then used to compare fibrinolysis in plasma with added aCAP compared to control plasma.

Fresh citrate plasma from a healthy volunteer was centrifuged (g=1600) for 20 minutes at room temperature. Plasma was mixed with 2160 µl 150 mM NaCl and 1105 µl control buffer (0.01 M Tris 0.1 M NaCl, pH 8) to a final volume of 4225 µl and a dilution of 1/4.4. Samples were placed in 15 glass tubes (650 µl in each) and all but three diluted 1/2 in 150 mM NaCl, yielding plasma dilutions of 1/8.8, 1/17.6, 1/35.2 and 1/70.4. To achieve
clot formation, 0.1 ml of human thrombin, 50 NIH units/ml (SIGMA Chemical company St. Louis, MO., USA), diluted in buffer (0.05 M Tris 0.05 M CaCl₂ 0.02% azid, pH 7.4), was added to each tube before incubation in water at 37°C for 15, 60 or 120 minutes. After centrifugation (g=1200, 7 minutes at room temperature) 400 µl of supernatant was mixed with 400 µl of aprotinin (4000KIE/ml in 150mM NaCl, Bayer Sverige AB, Sweden) to inhibit enzymatic activity. The mixture was immediately frozen at –20°C for later analysis of D-dimers using an ELISA assay, Asserachrom D-DI (Triolab, Diagnostica Stago, Sweden), with an inter assay CV of 5.4% (at 150 ng/ml) and 5.6% (at 994 ng/ml).

Fibrinolysis and aCAP: D-dimer measurements
Human fresh citrate plasma was centrifuged (g=1600) for 20 minutes at room temperature. By adding 2160 µl 150 mM NaCl and 1105 µl aCAP (1700 nmol/l) diluted in buffer (0.01 M Tris 0.1 M NaCl, pH 8), or controls of the same buffer, to a final volume of 4225 µl, a plasma dilution of 1/4.4 was achieved. Samples were diluted 1/2 with 150 mM NaCl, as described above, to a final plasma dilution of 1/35.2. Clot formation was achieved by the addition of 0.1 ml human thrombin 50 NIH units/ml (SIGMA Chemical company St. Louis, MO., USA), diluted in buffer (0.05 M Tris 0.05 M CaCl₂ 0.02% azid, pH 7.4), before incubation in water at 37°C for 15, 60 or 120 minutes. After centrifugation (g=1200, 7 minutes at room temperature), 400 µl of supernatant was mixed with the same amount aprotinin solution (4000KIE/ml in 150mM NaCl, Bayer Sverige AB, Sweden) and frozen until analysis of the D-dimer concentration as mentioned above.

Agarose fibrinogen gels
For preparation of gel, agarose, sheep fibrinogen, bovine thrombin and human plasmin (SIGMA Chemical company St. Louis, MO., USA) and buffer (0.1 M Tris, 0.05 M CaCl₂, pH 7.4), was used throughout the experiment. Fibrinogen and thrombin were dissolved in water.

Initially, 25 ml 1% (w/vol) agarose gel was heated to 100°C and at 65°C 2 ml 1% (w/vol) fibrinogen and 250 µl (25 NIH units) thrombin solution were added. A gel (1mm) was cast between glass plates. After 15 minutes the upper glass plate was removed and ten wells punched in the gel.

Different concentrations of aCAP (i.e. 3280, 1640, 820 and 410 nmol/l), 10 µl, were applied in two wells each (wells 2–9), and 10 µl of buffer in two control wells (well 1 and well 10). The gel was incubated in 37°C for 3 hours in a humid chamber. Buffer, 10 µl, was added to four wells with different concentrations of aCAP (wells 2–5) and to one control well (well 1). To achieve fibrinolysis, plasmin, 250mg/l, 10 µl, was added to the remaining wells (wells 6–10).

After incubation in a humid chamber (37°C) overnight, the gel was dried in 65°C for 15 minutes. Staining with 0.3% Coomassie (Coomassie brilliant blue R-250, BDH Laboratory, England) diluted in a solution of 5.95 mol/l ethanol and 1.75 mol/l acetic acid was performed before washing in the 5.95 mol/l ethanol and 1.75 mol/l acetic acid solutions. Fibrinolysis was visualized as the clear diameter around the wells.

Statistical methods
SPSS 15.0 (SPSS Inc., Chicago, USA) was used for calculation of Spearman’s correlation coefficient when concentrations of aCAP and the diameter of the fibrinolytic circle were compared. A p-value <0.05 was considered significant.
Results, Paper IV

Fibrinolysis model:
D-dimer measurements

Clot formation was seen visually after incubation in all dilutions at all timepoints. D-dimer levels increased with time in all dilutions. Levels of D-dimer increased with increasing plasma dilution up to a dilution of 1/35.2 but were somewhat lower for a dilution of 1/70.4 as shown in Figure 10. Since a dilution of 1/35.2 showed the best gradual increase of fibrinolysis over time, this was chosen for further experiment and comparison with aCAP.

Fibrinolysis and aCAP:
D-dimer measurements

Levels of D-dimer were lower in all samples containing aCAP as compared to corresponding controls and these differences increased over time, results shown in Figure 11.
Agarose fibrinogen gels

In wells without plasmin (number 1–5), no fibrinolysis could be seen, Figure 12. In the wells with plasmin and control buffer (number 10) a fibrinolytic circle of 10 mm in diameter occurred. The diameter of fibrinolysis was reduced proportionally with increasing concentrations of aCAP (wells number 6–9), Figure 12. The Spearman’s correlation coefficient for the relation between concentration of aCAP and the size of the fibrinolytic circle was 1 ($p<0.0001$).
General discussion

Methodological considerations

The measured levels of enzymes in serum and urine mirrors the pathophysiologic events in patients but must be considered with respect to biological and methodological factors. For measurements of aCAP, cross reactivity with proCAP might affect the results. This problem is decreased by diluting samples and measuring levels on the lower side of the standard curve where the cross reactivity is less pronounced. Used as a semi-quantitative assay, as in paper II, this cross reaction is probably of minor importance. In paper III, where levels are followed over time, cross reactivity might affect the lower levels to some extent, but measured levels of ir-aCAP is probably a good assessment of true aCAP levels.

Detection of ir-aCAP in serum could represent the enzyme activity, but the activity might be lost even though the antigen parts are intact. A common form of biological inactivation is the complex binding of an inhibitor to the enzyme. However, no signs of aCAP-inhibitor complexes could be detected in paper I.

The assay for trypsinogen 2 shows some cross reactivity (approximately 10%) with trypsin-2-α-1PI complexes [64], but this may be regarded as acceptable. Since the levels of trypsinogen 2 and formation of trypsin-2-α-1PI complexes increases more compared to trypsinogen 1 and corresponding trypsin-1-α-1PI complexes in AP, and perhaps to a larger extent in severe AP, levels measured in the trypsinogen 2 assay do not only reflect pancreatic leakage, but also other biological events [42,46]. The mechanism behind elevation of trypsinogen 2 in AP and severe AP is not fully understood, but the results with higher levels in severe compared to mild AP are in accordance with earlier studies [42].

When comparing patients with mild and severe AP, misclassification of patients can give misleading results. The Atlanta classification used in paper II and III can be questioned since it classifies patients with local complications such as pseudocysts, also without organ failure, as severe. This classification is, however, the latest consensus and is useful in comparing different clinical studies. All clinical notes where retrospectively reviewed by two different investigators who agreed on the classification of patients, thus limiting the risk of misclassification.

The results in paper II and III are mainly based on the measured levels of markers. Since the inter-assay CV for the methods used are low, the reproducibility is considered to be high.

For logistical reasons, it was not possible to include all patients with AP meeting the inclusion criteria in the studies described in paper II and III. This could result in a selection bias affecting the results. However, the representativity analysis showed that the group of included patients was similar to all other patients with AP during the same time period in Malmö.

Levels of markers might vary with patient related factors that are potentially associated with the risk of AP or severe AP, e.g. age, gender and aetiology. Patients in the severe group were somewhat older compared to the ones in the mild group, but stratification for patients younger and older than 70 years yielded similar results in the ROC analysis. The possible confounders age, sex and aetiology, were adjusted for in the logistic regression analysis and ought not to have affected the results.

The limited number of patients with severe disease implies a low statistical power for subgroup analysis, and the results in these analyses should be considered with caution. In the time course analysis low number of patients
Protease activation and inflammation in acute pancreatitis

with severe AP, especially seen for amylase, indicates a high risk of type II error, i.e. the risk of false negative results.

When comparing differences in median values between the mild and severe groups for each marker on Day1 to Day 3 in the time course groups and the whole study population, the patterns regarding significant differences (p<0.05) were similar for all markers except amylase. This indicates that the group used for time course analysis was representative of all patients in the present study.

No power calculation was performed since this was the first explorative study for these variables, and expected levels of differences between patients with mild and severe AP were not known.

**Activation of proenzymes in AP**

Activation of pancreatic proenzymes is an important event early in the course of AP. Since levels of activation peptides are higher in patients with severe AP it has been suggested that the active enzymes play a part not only in initiation of AP, but also in the development to severe AP [34,39,43]. The suggested pathophysiological effects of activated pancreatic enzymes have been hard to prove. Trypsin is the most investigated enzyme and its proteolytic mechanism could interfere in several biological systems. However, its duration of activity is presumed to be short because it is well regulated by inhibitors, and free trypsin is difficult to measure in plasma and urine [40,64]. Instead, many of the pathophysiological effects seen might come from other cascade systems, activated by trypsin. Since other proenzymes are activated by free trypsin, their activation is an indirect measurement of the initial trypsin activity. The effects of the active enzymes could also be of importance for pathophysiological mechanisms in AP.

**Measurement of aCAP**

The development of an assay for measurement of aCAP (described in paper I) made it possible to compare levels of pancreatic leakage, enzyme activation and activation peptide for the same enzyme, i.e. carboxypeptidase B and thus compare these pathophysiological events in patients with mild and severe AP (as done in paper III).

Although the aCAP ELISA shows a small crossreaction with proCAP, it can be used as a semiquantitative assay for measurements of aCAP. In patients with AP, levels of ir-aCAP were found to be high. Characterization by gel filtration confirmed most of the immunoreactivity in fractions corresponding to the size of aCAP. Surprisingly, there were no signs of activity for another complex that would suggest the presence of aCAP and a protease inhibitor, neither in samples from patients with AP nor in serum incubated with pure aCAP.

**Fibrinolysis, ischemia and aCAP**

The exact level of aCAP in pancreatic juice in AP is not known, but it could be roughly estimated to 1000–4000 nmol/l using information from other studies [70,156–159]. Enzymes with the same proteolytic mechanism as aCAP are known to inhibit fibrinolysis in concentrations much lower than this [35]. In paper IV, an inhibitory effect on fibrinolysis by aCAP is shown in two different experimental models. In the first model all plasma components are available and fibrinolysis is measured indirectly by increasing levels of D-dimers. In the second a direct effect on plasmin is shown eliminating possible false results caused by degradation of D-dimers. Formation of microthrombi in pancreas is seen in severe AP. Ischemia and impaired microcirculation is thought to have direct effects on the pancreatic gland by contributing to development of necrosis [29,103,104,113].
A suppression of fibrinolysis might increase the ischemic injury, further aggravating formation of necrosis and thereby the inflammatory reaction. An impaired pancreatic circulation also reduces supply of trypsin inhibitors and antioxidants which may be of importance for local and systemic progression of the disease.

**Leakage of pancreatic enzymes**

The local inflammatory reaction in the pancreatic gland causes a leakage of pancreatic enzymes and proenzymes into the circulatory system. Turnover for different enzymes in serum varies which may be a result of changes in leakage, production, or increased consumption, or differences in half-life in blood. Whereas leakage of not activated pancreatic enzymes makes them useful for diagnosis of AP, most of them do not correlate to severity of disease. Trypsinogen 2 differs from the other markers by its higher levels in severe disease making studies of its time course of interest [42,46]. The leakage of proCAP was measured in order to compare it with the timecourse of aCAP and CAPAP and thereby study possible differences in patterns of leakage and activation between patients with mild and severe disease.

**Inflammatory reactions**

The connection between protease activation and inflammatory response in AP is not well understood. In later phases the systemic inflammation in severe AP seems to occur totally independent from the proteolytic activity. Organ failure and mortality is often associated with this generalised inflammatory reaction [14,15]. In the first days of disease there is discrimination between patients keeping the localized inflammation in and around pancreas and patients developing a severe disease.

Many of the inflammatory mediators measured in AP correlate with disease severity but the complex inflammatory cascade is difficult to overview. The flora of regulatory mediators, feedback systems and parallel signalling ways does not make it easy to understand the pathophysiology and which mediators to modulate to gain amelioration of the disease [24,26,160]. Many studies in experimental AP are delicate to interpret since they are based on a model for mild or severe AP not always similar to the clinical situation. Biological and environmental differences between animals and humans also have to be considered.

Information gained from genetical studies might help understanding which mediators are of importance for human AP and development to severe AP. A genetic polymorphism resulting in increased production of MCP-1 increases the risk of getting severe AP, making it an interesting inflammatory factor [102]. MCP-1 also seems to be induced early in the course of disease, and is supposed to have pathophysiologic effects in early leukocyte recruitment [99,161]. Furthermore it has been shown to have the ability to trigger mast cells to exocytose TNF-α and might thereby generate a quick inflammatory response [162].

**Time course analysis**

In paper III, levels of MCP-1 increased early, already within 24 hours in patients with severe AP, and they seemed to stay elevated longer compared to markers of protease activation.

Markers of protease activation (aCAP and CAPAP) also increased within the first day in patients with severe AP, but levels of aCAP decreased quickly whereas levels of CAPAP decreased after 48 hours. In the timecourse analysis, aCAP did not show a longer half-life in serum compared to CAPAP, as was suggested because of its larger size and from earlier studies in pig without AP [33]. The elimination of aCAP is not thoroughly studied. It might be affected by other proteolytic
enzymes and, depending on their levels, to a higher extent in severe AP. Proteins with a molecular mass lower than 50 kDa, like proCAP, aCAP and CAPAP are normally eliminated through glomerular filtration and variable tubular degradation, resulting in small amounts of the native substance in the urine [163]. In an earlier study, large amount of CAPAP (10 kDa), but no proCAP (45 kDa) was recovered in urine from patients with AP [32]. In earlier studies in pig without AP aCAP was not excreted in urine [33]. Patients with severe AP might excrete aCAP in urine leading to a faster turnover; in fact, in our preliminary results aCAP was measurable in urine from patients with AP (unpublished results).

Leakage of pancreatic enzymes (proCAP, trypsinogen 2 and amylase) showed initially higher levels, decreasing with time. There were no significant differences between severe and mild disease, except for trypsinogen 2, which is in accordance with earlier studies [19,46,64,164].

The results from the time course analysis could be of importance when designing treatment studies. If inhibition of proteases should be expected to have effect, inhibitors have to be instituted very early, preferably within 24 hours from onset of symptoms. Also moderation of the earlier steps in the inflammatory cascade probably needs to be instituted already within 24–48 hours from onset of symptoms, before it has escalated.

**Severity prediction**

To do proper clinical treatment studies we need a marker of severity that is useful already within hours after onset of symptoms. The differences in median or mean levels seen between mild and severe patients in a study with pathophysiologic approach does not always discriminate severe from mild patients when a cut off level has to be set.

In paper II prediction of severity of AP on admission was studied. Median time from symptom onset was 24 hours, with a range of 4–72 hours, meaning that the admission sample for a specific patient could have been obtained on the first, second or third day from onset of symptoms.

MCP-1 exhibited to be a good marker of severity and CAPAP and aCAP to be usable markers of severity, even though they did not reach the predicting capacity of MCP-1 in this study. Since time from onset of symptoms at admission ranged from 4–72 hours the differences in levels on day 2–3 (described in paper III) might explain the lower prediction capacity seen for aCAP compared to CAPAP. Variability in time from onset of symptoms until admission might also explain differences in prediction capacity for CAPAP between this and earlier studies.

Since cut off values where set from the ROC curve based on the study material a prospective study with preset cut off values could result in somewhat lower prediction capacities for MCP-1 and aCAP. The cut off for CAPAP (2 nmol/l) in this study was in accordance with suggestions from an earlier study where higher CAPAP levels also showed to be useful in predicting severe AP [19].
Conclusions

- The ELISA for measurement of aCAP is an acceptable method for determining levels of aCAP and the levels of aCAP are elevated in serum from patients with AP.

- MCP-1 alone at hospital admission could be a good predictor of severe AP. MCP-1 in urine, CAPAP in serum and urine and aCAP may also be useful for prediction of severe AP.

- Markers of protease activation and inflammation are elevated early in severe AP, indicating that these processes are of pathophysiologic importance for development of severe AP within 24 hours from onset of symptoms. Activation of proteases seems to decrease rapidly after 48 hours.

- Fibrinolysis is inhibited by aCAP in vitro and may contribute to the development of pancreatic necrosis.

Implications for future studies and treatment of acute pancreatitis

Most clinical trials for pharmacological treatment of severe AP has provided disappointing results [88,165,166]. While enteral nutrition and early ERCP might lead to better outcome for groups of patients with severe AP, no clear effects have been shown for treatment with protease inhibitors, PAF antagonist or antioxidants [88,89,120,125,165–169]. One reason for this might be the timing of treatment, and inclusion of mild cases in previous studies [89,120]. As shown in the present thesis, markers for inflammation and protease activation reach high levels within the first day from onset of symptoms. In most studies, the time between first symptom and institution of treatment are much longer. Earlier institution of pharmacological treatment might give other results in the future. With a reliable early marker for prediction of severity and earlier admission to hospital, patients with potentially severe AP could be included in studies and treated within 24 hours from onset of symptoms, thus being more likely to benefit from a given treatment.
Acknowledgements

I would like to express my sincere gratitude to everyone who has supported me while working on my thesis, and especially to:

Anders Borgström, former professor at the department of Surgery and my principal supervisor for more than seven years, who passed away in October 2007. For supporting my interest in research, letting me keep my enthusiasm and try my ideas with a respectful, gentle guiding through the field of biochemistry, proteases and acute pancreatitis.

Jonas Manjer, former assistant supervisor and present principal supervisor, for giving me excellent discussions, constant support, epidemiological and statistic expert guidance and substantial advice.

Stefan Appelros, assistant supervisor and the one who got me interested in acute pancreatitis. For many interesting discussions, valuable comments, enthusiasm and constant support during this journey.

Professor Bengt Jeppsson for support, guidance and valuable comments.

Anne-Marie Rohrstock for your excellent laboratory guidance and work and for archiving all experiment ever done in the lab and furthermore, for being such a nice person.

Cleas Jansen for co-authorship and for sharing interesting results and thoughts.

Jalal Sadic for co-authorship, encouragement and optimism.

Johan Malm for co-authorship, valuable remarks and comments.

Björn Lindkvist, for practical as well as theoretical advice, support and for sharing your wise ideas concerning protease activation.

The Pancreas 2000 project, for education in pancreatology, leadership and research and especially for involving me in the network of pancreatologists.

My colleagues at the department of Surgery for encouragement and support.

The nurses and assistant nurses at the Department of surgery for their helpfulness in collecting the blood and urine samples.

My understanding friends, for being there even when I do not have time to see you.

My parents-in-law for help in taking care of our children and home, enabling me to spend more time with my research projects.

My parents for always supporting me, encouraging me and being there whenever I needed you. For taking care of our children with long or short notice.

Jonas, my wonderful husband, “the new and upgraded version”. For all love and understanding, for taking care of family and home and also for 24 hours a day technical and computer support whenever I needed it!

Stina and Max, for opening my eyes into other worlds and for enriching my life so much!

Our future baby, for keeping me on track, helping me to remember my deadline and for looking forward to the spring!
Bakgrund och målsättning


Bukspottkörteln har två huvudsakliga funktioner. I den inre sekretoriska funktionen ingår till exempel produktion och insöndring av insulin för reglering av blodsockernivån. Den yttre sekretoriska funktionen innefattar produktion av matsmältningsprotein (enzym) och utsöndring av dessa till tolvfingertarmen. Eftersom en del av dessa enzym har förmåga att bryta ner andra protein och därmed påverka viktiga biologiska processer, produceras de i en inaktiv form (proenzym) och aktiveras först när de når tolvfingertarmen där de bidrar till att spjälka födan.

De två viktigaste orsakerna till uppkomst av akut bukspottkörtelinflammation är gallstenssjukdom och överkonsumtion av alkohol. Däremot är det oklart på vilket sätt dessa faktorer leder till den cellskada och inflammation som uppstår i bukspottkörteln vid sjukdomen. De matsmältningsenzym som bildas i körteln har sannolikt en stor betydelse eftersom man noterat en för tidig aktivering av proenzym vid bukspottkörtelinflammation. Vid den milda formen av sjukdomen ses ett läckage av framför allt proenzym till blodbanan medan man vid den svårare formen av sjukdomen i högre grad sett tecken till aktivering av dessa enzym. Det mest studerade enzymet, trypsin, tros kunna påverka många olika biologiska system genom att förstöra eller aktivera viktiga reglerande proteiner i de biologiska processerna. Trypsin har också förstås att aktivera alla andra inaktiva bukspottkörtelenzym vilket sannolikt har betydelse för sjukdomsprocessen vid akut bukspottkörtelinflammation och kanske framför allt för utvecklandet av den svåra formen av sjukdomen. Man vet inte exakt hur aktiva bukspottkörtelenzym påverkar de biologiska systemen i kroppen, men i och runt bukspottkörteln tros diverse vävnadsskador som om kroppen bryter ner sig själv inifrån. Vävnads- skadan orsakar lokal inflammation som bidrar till sjukdomen.

Övergången från lokal inflammation i och runt bukspottkörteln till systemisk inflammation som drabbar även kroppens övriga organ har sannolikt stor betydelse för utveckling av den svåra formen av sjukdomen. Flera olika ämnen i denna komplexa process har studerats mycket de senaste åren. Dock är det inte klarlagt vad som styr utvecklandet av svår sjukdom. Sambandet mellan aktivering av bukspottkörtelenzym och inflammation är också oklart. En annan faktor som har betydelse för sjukdomsutvecklingen är blodförsörjningen till och i bukspottkörteln. Nedsatt blodförsörjning kan bidra till den process med vävnadsdöd som ses vid svår inflammation.
Ett stort problem i omhändertagande av patienter med akut bukspottkörtelinflammation är att det är svårt att tidigt skilja ut vilka som kommer att utveckla en svår respektive en mild sjukdom. Detta gör det också svårt att undersöka nya behandlingsmetoder för patienter med svår sjukdom. Då sådana behandlingar sannolikt har bäst effekt i ett tidigt skede av sjukdomen är det av största vikt att hitta en markör som tidigt visar patientens risk att utveckla svår inflammation.

Huvudsyftet med denna avhandling var att undersöka enzymaktivering och inflammation i tidiga skeden av akut bukspottkörtelinflammation och att identifiera skillnader mellan svåra och milda former av sjukdom.

Syften för de olika delarbetena var:

- Att utveckla en metod för bestämning av ett aktivt enzym, aktivt carboxypeptidas B i serum.

- Att undersöka förmågan hos olika markörer representerande enzym aktivering och inflammation att förutspå uppkomst av svår respektive mild bukspottkörtelinflammation.

- Att studera förloppen av sjukdomsprocesserna läckage av inaktiva enzym, aktivering av enzym och inflammation under de första tre dagarna hos patienter med mild respektive svår bukspottkörtelinflammation.

- Att undersöka om aktivt carboxypeptidas B hämmar nedbrytning av blodproppar.

**Metoder och resultat**

Syftet med det första arbetet var att ta fram en metod för bestämning av det aktiva bukspottkörtelenzymet aktivt carboxypeptidas B i serum. För att göra detta renades proteinet fram från bukspott och antikroppar mot detta protein framställdes. Dessa användes för att utveckla en så kallad ELISA metod för analys av aktivt carboxypeptidas B.

Aktivt carboxypeptidas kunde mätas i serum från patienter med akut bukspottkörtelinflammation. Trots undersökning med olika laborativa metoder kunde inte några tecken till hämning av aktivt carboxypeptidas B påvisas.

I delarbete två utvärderades förmågan att förutspå svår akut bukspottkörtelinflammation vid ankomst till sjukhus för markörer för enzym aktivering och inflammation. Både aktivt carboxypeptidas B och den aminosyresekvens som avspjälkas vid aktiveringens (CAPAP), och således representerar samma aktivitet, undersöcktes. Dessutom studerades en tidig, (Monocyte Chemoattractant Protein 1, MCP-1) och en något senare (C-reactivt protein, CRP), markör för inflammation. MCP-1 visade sig i denna studie ha den bästa förmågan att skilja mellan patienter som utvecklade svår respektive mild sjukdom redan vid ankomst till sjukhus. Såväl aktivt carboxypeptidas B som CAPAP visade sig också vara användbara för detta.


**Slutsatser**

- Aktivt carboxypeptidas B kan mätas i serum från patienter med akut bukspottkörtelinflammation. Inga tecken till hämmare kunde påvisas.

- Höga nivåer av MCP-1 vid ankomst till sjukhus kan förutspå vilka patienter som kommer att utveckla svår respektive mild akut bukspottkörtelinflammation. Aktivt carboxypeptidas B och CAPAP kan också vara till hjälp för tidig prognostisering av patienter med akut bukspottkörtelinflammation.


- Aktivt carboxypeptidas B hämmar nedbrytning av blodproppar vilket kan ha betydelse för blodcirculation och uppkomst av vävnadsdöd i bukspottkörteln vid svår akut bukspottkörtelinflammation.
References


22. Leach SD, Modlin IM, Scheele GA, Gorelick FS: Intracellular activation of digestive zymogens in rat pancreatic acini. Stimulation


Protease activation and inflammation in acute pancreatitis


87. McKay CJ, Curran F, Sharples C, Baxter
Protease activation and inflammation in acute pancreatitis


Protease activation and inflammation in acute pancreatitis


