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## Crosstalk between Inflammation and Coagulation in Acute Pancreatitis - Experimental and Clinical Studies.

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LUND UNIVERSITY

PO Box 117  
221 00 Lund  
+46 46-222 00 00



# Crosstalk between Inflammation and Coagulation in Acute Pancreatitis

Experimental and Clinical Studies

Ellen Andersson, MD

Department of Surgery,  
Clinical Sciences Lund  
Lund University, Sweden

Doctoral thesis 2010



**LUND UNIVERSITY**  
Faculty of Medicine

© Ellen Andersson, MD, 2010  
Contact:           ellen.andersson@med.lu.se  
                      skalpellen@hotmail.com

Supervisor: Professor Roland Andersson  
Department of Surgery, Clinical Sciences Lund  
Lund University Hospital, Sweden

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Nunc cognosco ex parte.

*1 Corinthians 13:12*

Il faut cultiver son jardin.

*Voltaire*

To Hugo



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# ORIGINAL PAPERS

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

- I. Andersson E, Axelsson J, Pedersen LC, Elm T, Andersson R. Treatment with anti-factor VII in acute pancreatitis in rats – blocking both coagulation and inflammation? *Scandinavian Journal of Gastroenterology* 2007;42:765-70.
- II. Axelsson J, Andersson E, Andersson R, Lasson Å. NFkB activation and anticoagulant treatment during acute pancreatitis in rat. *Journal of Organ Dysfunction* 2008;4:85-92
- III. Andersson E, Axelsson J, Andersson R. Treatment with active site inhibited factor VIIa - effects on inflammation in experimental acute pancreatitis. *Annals of Gastroenterology* 2010;23:180-86.
- IV. Andersson E, Ansari D, Andersson R. Major haemorrhagic complications of acute pancreatitis. *British Journal of Surgery*, in press. Epub Jun 16, 2010.
- V. Andersson E, Axelsson J, Eckerwall G, Ansari D, Andersson R. Tissue factor in predicted severe acute pancreatitis. *Submitted*.

# THESIS AT A GLANCE

	Question	Method	Result	Conclusion
I	Does prophylaxis with FVIIai reduce systemic inflammation in experimental severe AP?	TDC-induced AP, ELISA, enzyme Analysis 	Neutrophil recruitment in lung and ileum and levels of pro-inflammatory cytokines in plasma were reduced with FVIIai pre-treatment.	FVIIai pre-treatment in experimental severe AP inhibits systemic inflammation.
II	Does FVIIai prophylaxis affect activation of NFκB in remote organs during experimental severe AP?	TDC-induced AP, ELISA 	NFκB activation in lungs, ileum and liver was decreased by FVIIai pre-treatment.	NFκB activation in remote organs is reduced with FVIIai pre-treatment in experimental severe AP.
III	Does treatment with FVIIai reduce systemic inflammation in experimental severe AP?	TDC- induced AP, ELISA, enzyme Analysis 	Tissue neutrophil recruitment in lung was reduced with early FVIIai treatment.	Early FVIIai treatment exhibits some systemic anti-inflammatory effects in experimental severe AP.
IV	What is the incidence and outcome of major haemorrhagic complications in AP, and how are they managed?	Retrospective study of patients' charts 	14/1356 patients with AP had severe bleeding complications. 5/14 died. Angiographic intervention was successful in 1/3 patients. Two patients were subjected to surgery.	Major bleeding complications are rare, but clinically challenging and associated with high mortality.
V	Are levels of tissue factor (TF) altered in the early course of predicted severe AP and are TF levels related to severity?	Prospective clinical study, ELISA 	Levels of TF, in the early phase of predicted severe AP, are higher in patients with "true" severe AP than in those who develop mild disease.	High levels of TF early in the course of AP seems to be associated with severe AP.

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Akut pankreatit (AP) är en inflammation i bukspottskörteln, som oftast utlöses av gallstenssjukdom eller alkoholintag. Varför man får akut pankreatit vet man inte och inte heller varför de flesta patienter återhämtar sig på ett par dagar, medan ca 20 procent drabbas av en betydligt svårare form av sjukdomen. Vid svår AP ses en kraftig inflammatorisk reaktion i hela kroppen, där andra organ såsom lungor, njurar och lever kan drabbas och i värsta fall sluta fungera. Dödligheten i den svåra formen av AP är hög och likaså risken för resttillstånd, t ex i form av diabetes eller brist på matspjälkningsenzymer.

Att det finns ett samspel mellan inflammation och blodets levringsförmåga (koagulation) är känt sedan länge. I det normala fallet pågår en konstant balansakt mellan inflammation och anti-inflammation, och mellan koagulation och anti-koagulation. Vid svår inflammation kan denna balans dock rubbas. I de allvarligaste fallen kan inflammationen leda till ett tillstånd av bildning av små proppar i blodkärlen i hela kroppen, där ämnen som behövs för att blodet ska levera sig konsumeras. Den uppkomna bristen på material som behövs för att blodet ska levera sig leder till samtidiga blödningar på andra ställen i kroppen. Med olika sorters blodförtunnande medicin (anti-koagulantia) har man kunnat påvisa en samtidig hämning av inflammation, vilket avspeglar det nära samarbetet som pågår mellan dessa system. Hur samspelet mellan koagulation och inflammation ser ut vid AP och vilken effekt anti-koagulantia har på det inflammatoriska svaret vid AP är knapphändigt studerat.

I detta avhandlingsarbete har anti-koagulation i form av en inaktiverad form av koagulationsfaktor VII (FVIIai) använts. Den normala formen av koagulationsfaktor VII (FVII) cirkulerar i blodet i en inaktiv form, som aktiveras och startar blodkoagulationen då den binds till vävnadsfaktor (tissue factor). Tissue factor (TF) är en bindnings- och signaleringsanordning som uttrycks på cellytan på en rad celler belägna utanför blodbanan och som exponeras då ett blodkärl går sönder. Man har även sett att vissa celler involverade i kroppens försvar, såsom vita blodkroppar och celler på insidan av kärl, kan uttrycka TF vid inflammation. Dessutom har man sett att FVII och TF inte bara kan starta blodkoagulation, utan också kan vara inblandade i en rad andra funktioner i kroppen, såsom reglering av inflammation, cellers rörlighet och tillväxt av vanliga celler och cancerceller.

I de tre första delarbetena i denna avhandling studeras behandlingseffekten av FVIIai i en modell på råttor, som ges en mycket svår form av AP. I det första arbetet studeras effekten då FVIIai ges som förbehandling, det vill säga innan råttan får AP. Resultaten

visar att inflammationen lindrades i lungor och tarm samt att nivån av cirkulerande inflammatoriska ämnen i blodet (IL-6 och MIP-2) var lägre hos råttor som förbehandlats med FVIIai.

I det andra arbetet studeras huruvida aktiveringen av nuclear factor kappa B (NFκB) påverkas vid förbehandling med FVIIai hos råttor med svår AP. NFκB är en molekyl som finns i många av kroppens celler och som sköter reglering av hur gener uttrycks i cellerna, vilket i sin tur reglerar uttrycket av vissa inflammatoriska ämnen i kroppen. Resultaten visade att aktiveringen av NFκB var lägre hos råttor förbehandlade med FVIIai, vilket tyder på att hämning av NFκB utgör åtminstone en av verkningsmekanismerna för FVIIai:s inflammationsdämpande effekt.

I det tredje arbetet studeras effekten av efterbehandling med FVIIai, det vill säga behandling med FVIIai som ges till råttor som redan har svår AP. Detta scenario liknar mer verkligheten där man oftast inte kan behandla patienterna innan de blir sjuka. Resultaten visade en viss dämpad inflammation i lunga och en tendens till lägre nivåer av inflammatoriska ämnen i blodet. Effekten var dock betydligt mindre uttalad än när FVIIai gavs som förbehandling.

I det fjärde arbetet studeras förekomst av svåra blödningar hos patienter med AP. 1356 patientjournaler från 1994-2009 på alla patienter som vårdats i Lund med diagnosen AP gick igenom. Resultaten visar att det är mycket ovanligt med svåra blödningar. Bara 14 patienter med svåra blödningar hittades. Trots att det är ovanligt med svåra blödningar vid AP kunde man konstatera att dödligheten bland dessa patienter var mycket hög och att stora resurser i form operationer eller behandling via kärlröntgen (angiografi) ofta krävdes.

I det femte arbetet studeras nivåerna av TF i blodet hos 49 patienter med AP under de första tre dagarna på sjukhuset. Resultaten visar att TF är högre i gruppen av patienter som utvecklar en svår form av sjukdomen. Detta kan tyda på att TF är inblandat i utveckling av svår AP. När det gäller möjlighet att förutspå vilka patienter som kommer att få den svåra formen av sjukdomen visade sig nivån av TF vara bättre på att förutsäga detta än det kliniskt mest använda blodprovet, C-reaktivt protein (CRP), i prover tagna vid inkomst till sjukhuset.

Sammanfattningsvis dras slutsatserna:

- Att FVIIai har inflammationshämmande effekter i denna råttmodell av svår AP.
- Att NFκB-aktivering verkar vara en mekanism som påverkas av FVIIai.
- Att svåra blödningar hos patienter med AP är mycket ovanliga men svårbehandlade och riskfyllda.
- Att nivåerna av TF stiger tidigt vid AP och möjligen kan vara till hjälp för att förutsäga vilka patienter som kommer att utveckla svår AP.

# ABBREVIATIONS

AP	acute pancreatitis
APACHE	Acute Physiology and Chronic Health Evaluation
APC /PC	activated protein C / protein C
APTT	activated partial thromobplastin time
AT III	antithrombin III
Ca / Ca <sup>2+</sup>	calcium / calcium ions
CINC	cytokine-induced neutrophil chemoattractant
CRP	C-reactive protein
DIC	disseminated intravascular coagulation
ELISA	enzyme-linked immunosorbent assay
FDP	fibrin degradation product
FI-FXIII	coagulation factors, denoted by their Roman numerals
FVIIa	activated form of coagulation factor VII
FVIIai	active-site inactivated factor VII
EPCR	endothelial protein C receptor
ERCP	endoscopic retrograde cholangiopancreatography
IL	interleukin
NFκB	nuclear factor kappa B
MCP-1	monocyte chemoattractant protein-1
MIP-2	macrophage inflammatory protein-2
MPO	myeloperoxidase
PAI	plasminogen activator inhibitor
tPA / uPA	tissue type/urokinase plasminogen activator
PAF	platelet activating factor
PAR	protease activated receptor
PT-INR	prothrombin time - international normalised ratio
TAFI	thrombin-activatable fibrinolysis inhibitor
TDC	taurodeoxycholate
TF / asTF	tissue factor / alternatively spliced tissue factor
TFPI	tissue factor pathway inhibitor
TNF- α	tumour necrosis factor alpha
SIRS	systemic inflammatory response syndrome



# INTRODUCTION

Acute pancreatitis (AP) is an inflammatory process affecting the pancreatic gland. The spectrum of the disease ranges from a mild, self-limiting illness to a scenario of life-threatening multi-organ failure. Most patients presenting with AP will recover completely within a couple of days. In severe AP, however, the local inflammation is accompanied by a severe systemic inflammatory condition, potential pancreatic necrosis, organ failure, sepsis and death.

In literature, the history of acute pancreatitis (AP) starts in 1652, with one of the first known descriptions of the disease made by Nicholas Tulp, a Dutch physician and anatomist. Much progress in understanding the disease was made by Reginald Fitz, an internist and pathologist in Boston, who presented his work on AP in 1889. He described both clinical symptoms and pathologic findings of pus, necrosis and haemorrhage, as well as disseminated fat necrosis (Fitz 1889). His work reflects the wide variation in severity of AP.

Suggesting biliary reflux as the cause of AP, in 1901 Eugene Opie proposed that gallstone migration into the common bile duct was the cause of AP (Opie 1901). Opie's theory is still often cited, even though the harmful event is considered to be the increased pressure within the pancreatic duct, rather than the bile reflux *per se*. It was not until 1917 that alcohol was firmly established as an important pathogenetic factor in AP (Symmers 1917, Pannala et al. 2009). Progress in understanding the pathophysiology of the systemic engagement in AP was made by Rinderknecht in 1988. He hypothesised that an excessive stimulation of leukocytes, rather than circulating pancreatic digestive enzymes, was responsible for the systemic progression of the disease (Rinderknecht 1988).

In the past decades, focus in research on AP has been set on understanding the pathophysiologic mechanisms, improving intensive care, optimising diagnostic tools and finding early predictors of severity. Despite remarkable progress, our understanding of the pathophysiology and the ability to accurately predict the severity of AP remain limited, and the most effective remedies for AP are still purely supportive in nature. Although many different substances with versatile modes of action have demonstrated promising results in the experimental setting, the progress concerning specific clinical therapy has been limited. With many unresolved questions concerning the underlying pathophysiology and its enigmatic wide variation in clinical presentation, AP still remains a clinical and scientific challenge.



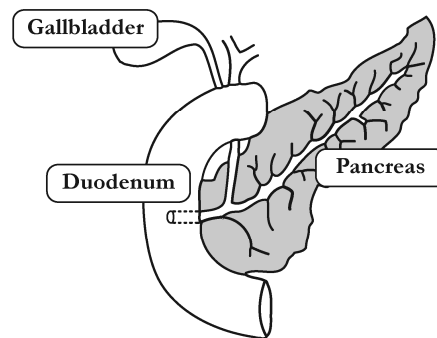


# GENERAL BACKGROUND

## Acute pancreatitis from a clinical perspective

Acute pancreatitis (AP) is an inflammatory process in the pancreas. It involves local inflammation of the pancreatic gland (Figure 1), but may progress into a systemic inflammatory response (SIRS) and failure of one or more distant organs (Bradley 1993).

The clinical diagnosis of AP is based on the combination of acute upper abdominal pain and elevated plasma levels of pancreas-specific amylase or lipase (Steinberg et al. 1994). Severe AP is defined by scoring systems based on clinical presentation (according to e.g. Ranson or APACHE II), evidence of organ dysfunction and intra-pancreatic pathology, such as the development of necrosis or pseudo-cysts (Bradley 1993).



**Figure 1.** *The pancreatic gland and its relation to the duodenum and the gallbladder.*

AP affects about 300 patients per  $10^6$  inhabitants and year. The cause of AP is in most cases gallstone disease or excessive alcohol consumption (Andersson et al. 2004, Appelros et al. 1999). Most patients present with a mild, self-limiting disease, with complete recovery within a couple of days. However, 15-20 percent will succumb to a severe form of AP, associated with multiple organ failure, sepsis and death. The rate of mortality in severe AP is reported to be in the range of 9-47 percent (Appelros et al. 2001, Gloor et al. 2001, Andersson et al. 2004, Gislason et al. 2004).

Two peaks in mortality are seen during the duration of illness. About 50 percent of deaths occur within the first week. The cause of these early deaths is considered to be

a severe initial attack of AP, followed by an excessive SIRS, concomitant multi-organ failure and death. The second peak in mortality occurs after about two to three weeks. These patients survive the first storm of severe systemic inflammation, but develop extensive pancreatic and peripancreatic necroses complicated by infection, sepsis and death.

Certain prognostic factors increasing the risk of severe AP and mortality, such as high age (Compañy 2003) and obesity (Martinez 2006), have been suggested. The answer to why some patients recover in a few days from a mild episode of AP, while others progress to severe disease is, however, still not known.

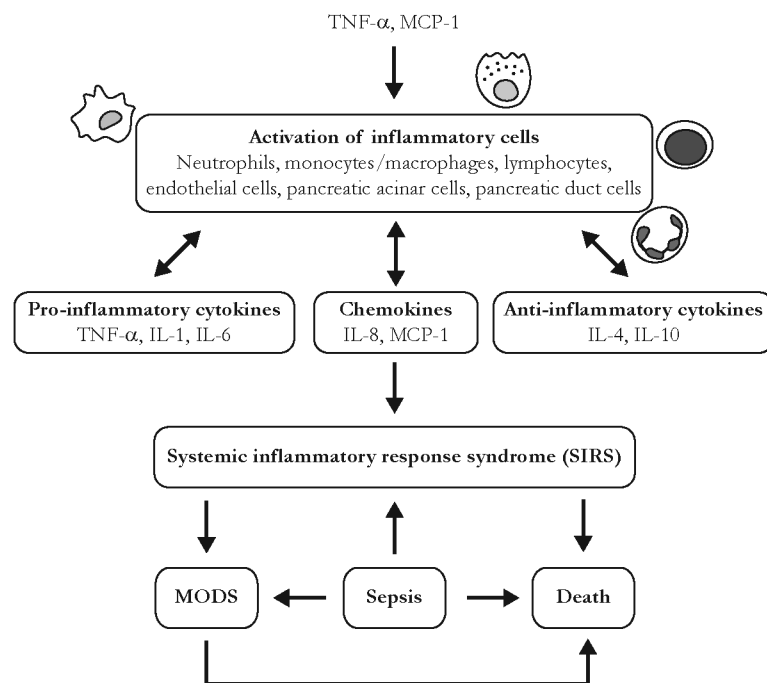
## Pathophysiology

The exact mechanisms eliciting AP are not clear. Regardless of aetiology, the most common theory is that AP starts by an injury to the pancreatic acinar cells, resulting in leakage of pancreatic enzymes into adjacent tissue. The activation of trypsinogen to trypsin plays a key role in this process, leading to cell injury in experimental models of acute pancreatitis and in experiments on isolated pancreatic acinar cells (Saluja et al. 1997 and 1999). Activation of proteases and lipase leads to auto-digestion of cells and stroma, eventually causing oedema, necrosis and vascular damage. Protective mechanisms preventing premature activation of the pancreatic enzymes include the synthesis of trypsin as an inactive enzyme, trypsinogen, autolysis of activated trypsin, enzyme compartmentalisation, synthesis of specific trypsin inhibitors and low intracellular ionised  $\text{Ca}^{2+}$  concentrations. Larger amounts of liberated trypsin, however, overwhelm the defence mechanisms of the pancreas, and activate other pro-enzymes.

After the initial pancreatic acinar cell injury, irrespective of the triggering factor, pathophysiologic steps seem to take a similar path for all patients with AP. The subsequent extra-acinar events consist mainly of a distinct immune response. Inflammation is initiated with local production of mediators such as  $\text{TNF-}\alpha$ , IL-1, IL-6, and IL-8, which are released from acinar cells, endothelial cells and pancreatic duct cells (Bhatia et al. 2001, Schafer et al. 2005). Circulating monocytes, neutrophils and lymphocytes are activated by pro-inflammatory cytokines, and adhesion molecules expressed by endothelial cells allow these cells trans-endothelial migration into the pancreatic interstitial space. These infiltrating inflammatory cells accelerate further production and secretion of cytokines. As a result, more leukocytes in the circulation and endothelial cells in the pancreas and in distant organs, such as the lung and the liver, are activated. This activation elicits further microcirculatory derangements, such as increased vascular permeability and accelerated leukocyte transmigration.

The progression of AP can be viewed as a three-phase continuum: local pancreatic inflammation, a generalised inflammatory response, and the final stage of sepsis, with multiple organ dysfunction (Figure 2). The disease process can extend to any of the three phases. The disease is often resolved after the local inflammatory process, resulting in mild AP. If the disease progresses injury in remote organs can be elicited by the release of inflammatory mediators from the pancreas and from extra-pancreatic organs, such as the liver and the lungs. A SIRS-scenario with remote organ dysfunction will result in severe cases, where respiratory failure tends to come first, followed by liver and kidney dysfunction, and eventually potential failure of all other organ systems, such as cardiac failure and gut barrier dysfunction. As a consequence of inflammation, local complications, such as acinar cell necrosis, pseudocyst formation, and abscesses can develop.

The patients surviving the first insult may die later by an initially minor infection, such as a line infection or pneumonia, that normally would not be life-threatening. According to this “two-hit hypothesis”, the initial overactive SIRS somehow primes the inflammatory response, leading to an exaggerated secondary SIRS and possibly death (Weber et al. 2001, Bhatia et al. 2000, Moore et al. 1995).



**Figure 2.** Pathophysiology of disease progression in acute pancreatitis.

## Inflammatory cells and mediators

Multiple cells are involved in elaboration of the inflammatory mediators in AP, the most important cells being the pancreatic acinar cells, the endothelial cells, the neutrophils, the lymphocytes, and the macrophages/monocytes. A variety of inflammatory mediators are engaged in the inflammatory process, where cytokines and free oxygen radicals released from different cells play prominent roles. They elicit responses resulting in modulation of leukocyte trafficking, increased vascular permeability, localised tissue destruction, and generalised inflammation, with damage to lung, kidney, and various other organs.

### *Monocytes/macrophages*

The mononuclear phagocyte system consists of leukocytes, whose primary function is phagocytosis. Monocytes are the circulating form, and once settled in tissue the monocytes mature and become macrophages. Monocytes/macrophages are “scavenger cells”, phagocytosing foreign particles, such as microbes and antigens, malfunctioning endogenous cells and cellular debris. Macrophages are also capable of producing cytokines and chemokines that recruit other inflammatory cells, especially neutrophils. The macrophage interacts with lymphocytes by presenting antigens on its surface, and lymphocytes may enhance the phagocytic efficacy of macrophages, by activating macrophages through cytokine release.

Resident macrophages seem to play a minor role during the initiating process of AP, but it has been shown that monocytes are rapidly recruited in several models of AP, including the caerulein (Adler et al., 1979) and taurodeoxycholate (TDC) (Axelsson et al., 2007) models of experimental AP in rats.

Monocytes cause tissue damage during AP by releasing reactive oxygen species. In contrast to neutrophils, targeted depletion of monocytes or its chemotactic agents, does not reduce the local pancreatic injury, but only ameliorates secondary effects such as AP-induced pulmonary injury (Gerard et al., 1997).

### *Mast cells*

The mast cell is resident in many tissues. It contains granules rich in histamine and heparin. Mast cells express tissue and urokinase plasminogen activator (tPA, uPA). The mast cell is a key component of the immune system and the normal inflammatory response. Its vicious potential is demonstrated in anaphylactic reactions. A very large population of mast cells resides in the periacinar space and pancreatic interstitium, and it degranulates early in acute pancreatitis, especially in the severe forms. Mast cells degranulation occurs within five minutes in lethal bile-salt

pancreatitis (Closa et al. 1994), but not for three to four hours in the mild caerulein-induced pancreatitis rat model (Shimizu et al. 1993). The release of mast cell mediators focuses inflammation and the enzymatic assault to the gland's interstitium and the peritoneal compartment. It has been speculated that mast-cell tryptase is responsible for the activation not only of trypsinogen, but also of other pancreatic pro-enzymes (Vanderslice et al. 1989, Braganza 2000). Mast cells can amplify lung damage by way of products with degranulating capacity that enter lymphatics and venules, and can activate blood coagulation (Colman et al. 1997, Valent et al. 1998). Mast-cell mediators may also be involved in fibrinolysis by the release of tPA and uPA without PAI (Valent et al. 1998). Activation of mast cells is involved in the development of endothelial barrier dysfunction in both the pancreas and extrapancreatic organs (Dib et al. 2002) and mast cell stabilizers prevent pancreatitis-induced pulmonary endothelial barrier dysfunction (Zhao et al. 2005).

### *Neutrophils*

Circulating polymorphonuclear leukocytes, or neutrophils, respond rapidly to chemotactic stimuli, and are able to phagocytose and destroy foreign particles. Neutrophils are recruited by the chemoattractant IL-8 in humans and mainly by CXCL2 (MIP-2) in rats. Once activated, primarily by cytokines produced by endothelial cells and macrophages, neutrophils are able to extravasate and migrate to sites of inflammation. They exert an important part of the immune response and release proteins such as myeloperoxidase (MPO) and elastase. MPO is an iron-containing heme protein localized in the azurophilic granules of neutrophils and in the lysosomes of monocytes involved in the killing of several micro-organisms and foreign cells, including bacteria, fungi, viruses, red blood cells, and malignant and non-malignant nucleated cells. Although it is expressed by other cells, it occurs in higher amounts in neutrophils.

Neutrophils are considered to be the predominant inflammatory cells inflicting damage to the pancreatic tissue. Depletion of neutrophils in caerulein-induced pancreatitis dramatically reduces severity and injury of the pancreatic parenchyma, and limits the extension of remote organ injury, e.g. in the lungs (Pastor et al. 2006). In a model of severe AP less pulmonary injury and increased survival rates were noted in neutrophil depleted rats (Inoue et al. 1995).

### *Lymphocytes*

Lymphocytes are capable of specifically recognising and distinguishing different antigenic determinants. The lymphocytes consist of B-lymphocytes, T-lymphocytes and natural killer cells. They may be further subdivided into functional subsets, defined by different surface antigens, "clusters of differentiation; CD". In response to

antigens, T-lymphocytes may recruit and activate other inflammatory cells, such as monocytes/macrophages and neutrophils. Lymphocyte infiltration into the pancreatic tissue occurs early in AP. Depletion of CD4<sup>+</sup> cells, but not depletion of CD8<sup>+</sup> cytotoxic T-lymphocytes, decreases pancreatic injury in caerulein-induced AP (Demols et al. 2000). This implies that the non-cytotoxic functions of lymphocytes, such as cytokine release and activation of other inflammatory cells, may be of importance. The mechanisms of action of CD4<sup>+</sup> lymphocytes in AP are, however, not clarified.

The aetiology of AP may predict the role of lymphocytes, since biliary-induced AP showed an increased number of circulating CD4<sup>+</sup>, while alcohol-induced AP did not (Bhatnagar et al. 2001). It has been demonstrated that there is a significant decrease in the number of total circulating lymphocytes and lymphocyte subset count relative to controls in the early phases of human AP (Pezzilli et al. 1995). An impaired early activation of a certain subtype of T-lymphocytes, CD19<sup>+</sup>, has been detected in patients with severe AP, when compared to patients with other causes of acute abdominal disease (Pezzilli et al. 2003). In patients with severe AP, a dramatic depletion of circulating natural killer cells was found along with a reduction of circulating CD3<sup>+</sup> and cytotoxic CD8<sup>+</sup> lymphocytes (Dabrowski et al. 2008).

### *Inflammatory cytokines and chemokines*

The cytokines constitute a group of soluble proteins that are released by different cells, causing a change in function or development of the cell itself (autocrine), an adjacent cell (paracrine), or a distant cell (endocrine). Cytokines are important messengers in a variety of cellular functions, including reproduction, growth and development, normal homeostatic regulation, response to injury and repair, blood clotting, inflammation and host resistance. The expression of cytokines is a well regulated process, modulated by transcription factors such as NFκB.

The chemokines represents a large family of small, structurally homologous cytokines. The chemokines are divided into four groups based on the cysteine sequence; CXC, CC, C, and CX3C (Rollins 1997). Many chemokines, such as IL-8, have been implicated in various aspects of the propagation of AP. Chemokines act as potent chemoattractants, predominantly exerting their effects on neutrophils, but they may also be involved in regulation of recruitment of T-lymphocytes. The chemokines bind to cellular transmembrane G-protein coupled receptors. Binding leads to increased intracellular calcium concentrations and activation of protein kinase C (Makhija et al. 2002).

The pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8, and platelet activating factor (PAF), the anti-inflammatory cytokines IL-4, IL-10, IL-11, IL-18, as well as TNF-α soluble receptors and IL-1 receptor antagonists, have been shown to be

intimately involved in the inflammatory response in AP (Makhija et al. 2002, Mayer et al. 2000, Granger et al. 2005). Important inflammatory cytokines and chemokines, and short descriptions of their roles in AP, are depicted in Table 1.

**Table 1.** *Inflammatory cytokines and chemokines in acute pancreatitis.*

Marker	Role in acute pancreatitis
TNF- $\alpha$	Pro-inflammatory. Neutrophil activation. Shock.
IL-1 $\beta$	Pro-inflammatory. Neutrophil activation. Shock.
IL-2	Pro-inflammatory? Raised levels in AP.
IL-4	Anti-inflammatory. Inhibits release of pro-inflammatory cytokines.
IL-6	Leukocyte activation. Acute phase response. Pyrexia. Correlate with severity.
IL-8	Neutrophil activation and chemotaxis. Correlate with severity.
IL-10	Anti-inflammatory. Inhibits release of pro-inflammatory cytokines.
IL-11	Anti-inflammatory. Ameliorate histological severity.
IL-18	Pro-inflammatory.
MCP-1	Monocyte chemoattractant. Produced by acinar cells early on induction of AP.
PAF	Pro-inflammatory.
Substance P	Pro-inflammatory

### *Interleukin 6*

IL-6 is one of the main inducers of acute phase proteins, such as C reactive protein (CRP) and fibrinogen synthesis in the liver, and peaks earlier than CRP. IL-6 is produced by a wide spectrum of cells, including monocytes/macrophages, neutrophils, lymphocytes, pancreatic acinar cells, pancreatic ductal cells, pancreatic stellate cells, vascular endothelial cells and fibroblasts in response to stimuli. Experimentally induced pancreatitis results in IL-6 release within the pancreatic tissue (Norman et al. 1997). Levels of IL-6 may serve as markers of severity of AP, the magnitude of the increase correlating with the course of the disease (Makhija et al. 2002). IL-6 has been demonstrated to induce coagulation in humans (Stouthard et al. 1996).

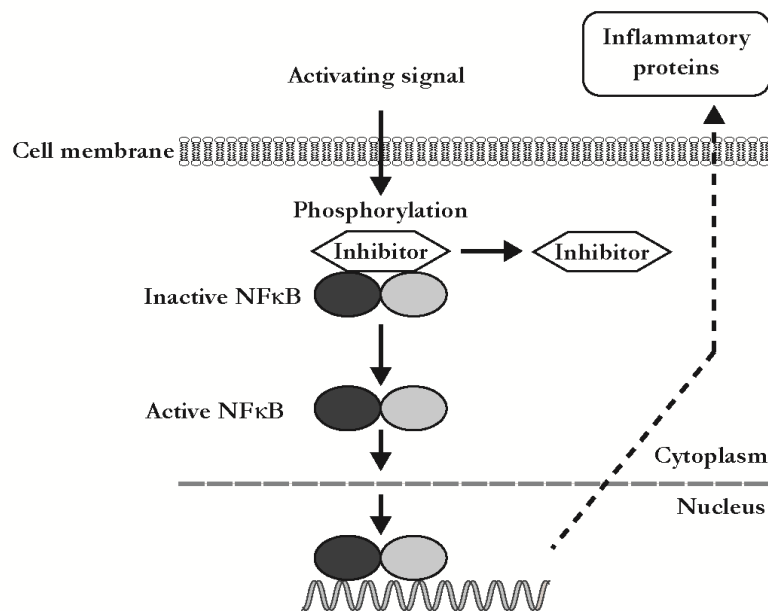
### *Macrophage inflammatory protein 2*

Four basic neutrophil chemotactic factors (chemokines) or cytokine-induced neutrophil chemoattractants (CINC:s) have been identified in inflammatory tissue in the rat, GRO/CINC-1, GRO/CINC-2  $\alpha$ , CINC-2  $\beta$ , and CINC-3 (Nakagawa et al. 1994). CINC-3 was previously called MIP-2. CINC:s show large sequential similarities to human IL-8, the most important human neutrophil chemoattractant (Nakagawa et al. 1994). Rats do not express IL-8. CINC:s are believed to play an important role in the systemic inflammatory response in AP, and anti-CINC

antibodies reduce the pulmonary injury associated with caerulein-induced AP in the rat (Bhatia et al. 2000).

### *Nuclear factor kappa B*

Increasing knowledge has been gained on the intracellular signalling pathways involved in the inflammatory response in AP, where nuclear factor kappa B (NF- $\kappa$ B), a nuclear transcription factor, is of importance. NF- $\kappa$ B has been suggested to be of importance in the initiation of AP, not only in pancreatic acinar cells and monocytes/macrophages but also in distant organs, such as the lung. Normally NF- $\kappa$ B is bound to inhibitors in the cytoplasm, but when cells are stimulated by cytokines or reactive oxygen species the inhibitor is phosphorylated and degraded and NF- $\kappa$ B may translocate into the nucleus and induce transcription of its target genes (Figure 3) (Barnes et al. 1997). NF- $\kappa$ B is able to control production of cytokines and adhesion molecules, as well as specific inducible nitric oxide synthase enzymes, mediating a variety of inflammatory events involved in AP (Rakonczay et al. 2008).



**Figure 3.** NF- $\kappa$ B activation in response to activating signals, such as cytokines. Once the cytoplasmic inhibitor is phosphorylated and degraded, NF- $\kappa$ B is released, activated and translocated into the nucleus, where it exerts its role as a transcription factor. (Adapted from Rakonczay et al. 2008).

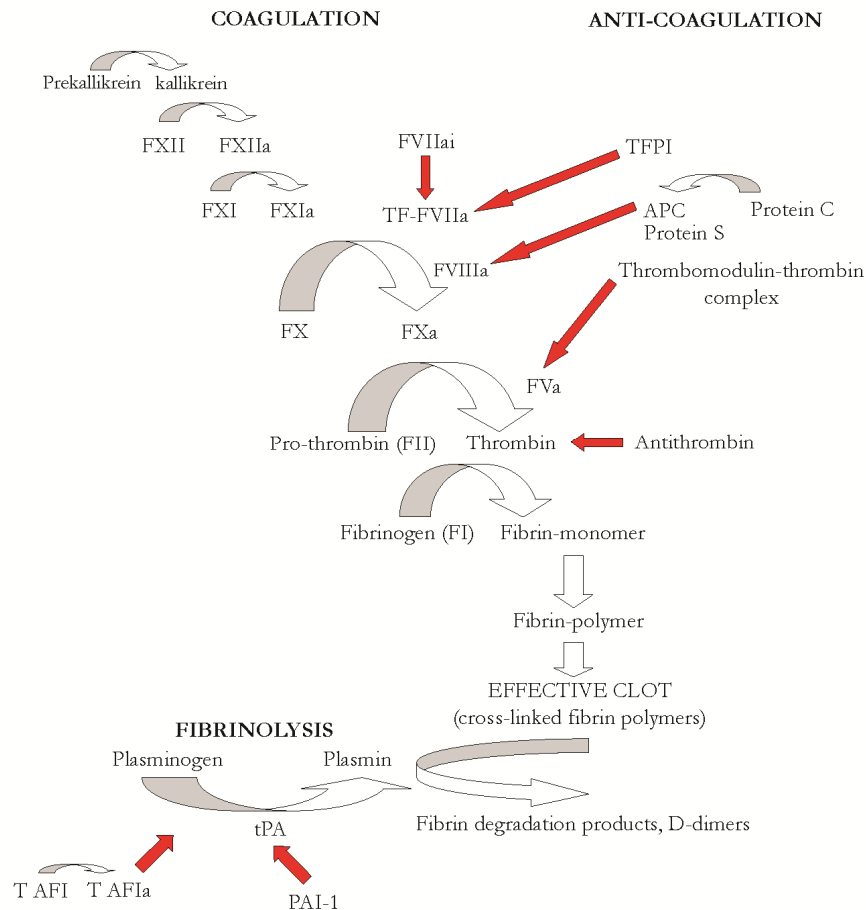


The correlation between localized NF- $\kappa$ B activation, cytokine up-regulation, and tissue damage in the pancreas suggests a key role for NF- $\kappa$ B in the development of the inflammatory response of AP (Vaquero et al. 2001). In caerulein-induced AP in the rat, NF- $\kappa$ B activation occurs in a biphasic manner. The first phase occurs after 30 minutes and the second phase after 3-6 hours (Gukovsky et al. 1998). In taurodeoxycholate (TDC)-induced AP in rats, nuclear translocation of the NF- $\kappa$ B p65 subunit has been demonstrated to be associated with up-regulation of adhesion molecules in acinar and endothelial cells, inducing transmigration of neutrophils (Telek et al. 2001). Activated neutrophils increase production of oxygen free radicals which further enhance pancreatic NF- $\kappa$ B activation (Rakonczay et al. 2008).

## **Crosstalk between inflammation and coagulation**

It is well established that systemic inflammation and coagulation are closely linked and it has been known for long that coagulopathy is associated with AP and related to overall morbidity (Ranson et al. 1977). In the worst cases, fully developed disseminated intravascular coagulation (DIC) can occur (Maeda et al. 2006). Initially a direct activation by micro-organisms or endotoxin was theorised to be the trigger of coagulation in inflammatory conditions, but nowadays cytokines are attributed the role of mediating activation of the coagulation system (Levi et al. 1997). Several studies have shown the importance of IL-6 in the initiation of coagulation, and the role of TNF- $\alpha$  and IL-1 in the regulation of physiological anti-coagulation (Van der Poll et al. 1994, Van Deventer et al. 1990, Boermeester et al. 1995). The tissue factor – factor VII (TF-FVIIa) pathway, previously referred to as the “extrinsic pathway” (Boermeester 2009, Mackman 2009), is recognised to be the pathway activating coagulation in response to inflammation (Figure 4).

Coagulation induced by inflammation is characterised by abundant intravascular fibrin deposition, resulting from enhanced fibrin formation and impaired fibrin degradation (Wheeler et al. 1999, Vallet 2001, Levi et al. 2009). This is caused by an increase in TF/FVIIa-mediated generation of thrombin, and depression of anti-coagulant mechanisms, such as protein C/protein S, tissue factor pathway inhibitor (TFPI) and the antithrombin system. Endogenous fibrinolysis is impaired by high levels of circulating plasminogen activator inhibitor (PAI)-1, which is the most important inhibitor of plasminogen activation. A key to the crosstalk between inflammation and coagulation are specific cellular receptors, such as protease activated receptors (PARs) on inflammatory cells and endothelial cells, through which activated coagulation proteases may induce signalling and thereby modulate the inflammatory response (Levi et al. 2003, Pawlinski et al. 2004).



**Figure 4.** The coagulation system and the fibrinolytic system. Dark arrows denote inhibitory effects.

Apart from coagulopathy and thromboses, other systemic micro-vascular disturbances including vasoconstriction, shunting, inadequate perfusion, and increased blood viscosity are seen in AP (Kinnala et al. 2001, Cuthbertson 2006). Larger vessels may also be affected by AP, but this usually occurs late in the course of the disease (Beattie et al. 2003). Major bleeding caused by acute rupture of vessels or pseudoaneurysm formation is a severe complication of AP, resulting from local severe inflammation and enzymatic insult to major pancreatic or peripancreatic vessels.

### *Experimental studies*

Several experimental studies confirm the involvement of coagulation, anti-coagulation and fibrinolysis, as well as micro-vascular dysfunction, in AP. In rats, AP is induced when polystyrene micro spheres are injected into the splenic artery to prevent perfusion of the splenic part of the pancreas. This supports the role of small vessel ischaemia in the pathogenesis of AP (Redha et al. 1990). In necrotising AP in rats, an increase of plasminogen activator inhibitor (PAI) activity and a decrease of plasminogen activator have been detected early on during the course of the disease, reflecting a depressed fibrinolysis (Rydzewska et al. 1992).

Studies of AP in dogs have shown increased platelet turnover and altered platelet aggregation (Jacobs et al. 1986, Lukaszuk et al. 1989). In another study on acute necrotising AP in dogs, decreased levels of platelets, plasminogen, and the natural anticoagulant antithrombin III (AT III) were detected, accompanied by raised levels of fibrinogen and increased international normalised ratio (INR) (Feldman et al. 1981), reflecting derangements in both coagulation, anti-coagulation and fibrinolysis.

Early activation of the natural anti-coagulant protein C has been demonstrated in a study in rabbits with necrotising AP (Ottesen et al. 1999).

A deficiency of FVII attenuates the acute inflammatory response in mice (Xu et al. 2006), confirming a role of FVII in the inflammatory reaction.

### *Clinical studies*

Tissue samples from patients operated on due to severe AP, show ultrastructural changes consistent with coagulation, such as accumulation of platelets intra- and extravascularly, fibrin deposition in the connective tissue and micro thrombi in blood vessels (Bockman et al. 1986). Upregulation of urokinase plasminogen activator (uPA), its membrane receptor urokinase plasminogen activator receptor (uPAR), and its inhibitor plasminogen activator inhibitor-1 (PAI-1), were found adjacent to the areas of necrotic tissue (Friess et al. 1998). Based on studies on septic patients, it may be speculated that the activation of fibrinolysis in the microcirculation is accompanied by increased inhibitors of fibrinolysis systemically. This leads to a decrease in fibrinolytic capacity in the circulation, which could result in deposition of fibrin in remote organs such as the lungs (Voss et al. 1990).

Other clinical studies have shown that plasma levels of fibrinogen, von Willebrand factor and the rate of platelet activation may be associated with the severity of AP (Kerekes et al. 2001). In a study in Soweto, an early profound systemic fibrinolysis was suggested as a possible explanation of the aggressive nature of alcoholic acute pancreatitis in this area (Segal et al. 2002).

### *Factor VII and tissue factor*

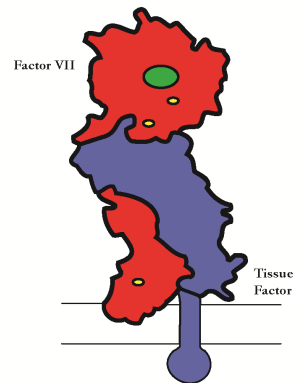
Factor VII and tissue factor are considered to be important in the cross-talk between inflammation and coagulation (Levi et al. 2006).

Factor VII (FVII) is a vitamin K-dependent trypsin-like serine protease, produced in the liver. Factor VII is the natural ligand of TF (Figure 5) and partner in haemostatic and non-haemostatic functions. The active form of factor VIIa (FVIIa) appears to exist in equilibrium between minor active and dominant zymogen-like inactive conformational states. Approximately 99 percent of FVII circulates as inactive zymogen. The binding of TF to FVIIa (Figure 5) is assumed to shift the equilibrium into the active state (Soejima et al. 2001). Activation of FVII involves protease cleavage of the zymogen to generate a 152-amino acid light chain and a 254-amino acid heavy chain linked by a disulfide bond. The light chain is aligned by the N-terminal 10-carboxyglutamic acid (GLA) domain, which in the presence of calcium enables reversible binding to negatively charged phospholipid membranes, the hydrophobic stack, which is involved in formation of the complex with factor X, and two epidermal growth factor (EGF)-like domains. The heavy chain of FVIIa contains the catalytic serine protease domain. FVIIa is an extremely weak serine protease on its own. Although activated, FVIIa does not express its full procoagulant functionality until it is bound to TF (Person 2006). The association of FVII with TF enhances the proteolytic activity of FVIIa by  $10^5$ . TF-FVIIa binding brings the binding sites for both the substrates (factors X and IX) and the enzyme (FVIIa) responsible for initiating coagulation closer to each other, and induces a conformational change of FVIIa (Ruf 1998).

Levels of FVII increase with age and are higher in hypertriglyceridemia (Rao et al. 1999). FVII has the shortest half-life of all procoagulant factors (3-6 h), whereas the half-life of FVIIa is relatively long (2.5 h) compared to other activated coagulation factors. A strong contribution of the FVII genotype to levels of FVII has been demonstrated, and different FVII genotypes can result in up to several-fold differences in mean FVII levels (Roberts et al. 2001)

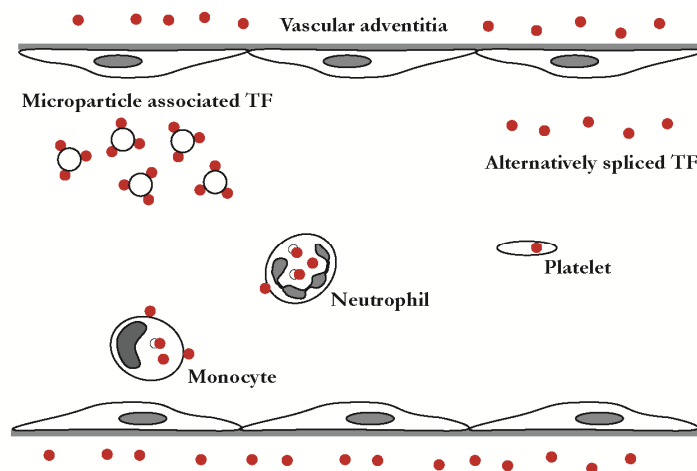
Tissue factor (TF) is a 263-amino acid trans-membrane glycoprotein, consisting of an extracellular domain, a transmembrane domain, and a cytoplasmic tail (Figure 5) (Daubie et al. 2007). In the vessel wall, TF is found in vascular smooth muscle cells, adventitial fibroblasts, and pericytes (Wilcox et al. 1989). The distribution of TF in different organs varies, and is non-uniform within single organs, with a cell-type specific distribution. Highly vascularised organs, such as lung, brain and placenta demonstrate high levels of TF, whereas skeletal muscle and cardiac muscle show very low levels of TF (Drake et al. 1989, Flössel et al. 1994). In endothelial cells TF is normally not expressed, but can be induced by pro-inflammatory cytokines (Cirillo et al. 2005). After binding to their corresponding cytokine-receptor, intracellular signalling is initiated through the MAP-kinases p38, ERK, and c-jun terminal NH<sub>2</sub>-

kinase, with downstream activation of transcription factors such as NF- $\kappa$ B, AP-1 or EGR-1. Also the protein kinase C, as well as the Rho-Kinase pathway, is known to mediate TF induction (Breitenstein et al. 2010).



**Figure 5.** Simplified molecular model of TF (lighter) and FVII (darker).

TF was initially defined as an initiator of coagulation, triggering the coagulation cascade by formation of the “ternary complex”, a complex consisting of TF, FVIIa and zymogen factor X, which then is activated to factor Xa (FXa). The latter cleaves prothrombin into thrombin, which turns fibrinogen into fibrin monomers. During the past two decades TF has been recognised to have many other non-haemostatic roles in inflammation, tumour growth and angiogenesis (Daubie et al. 2007).



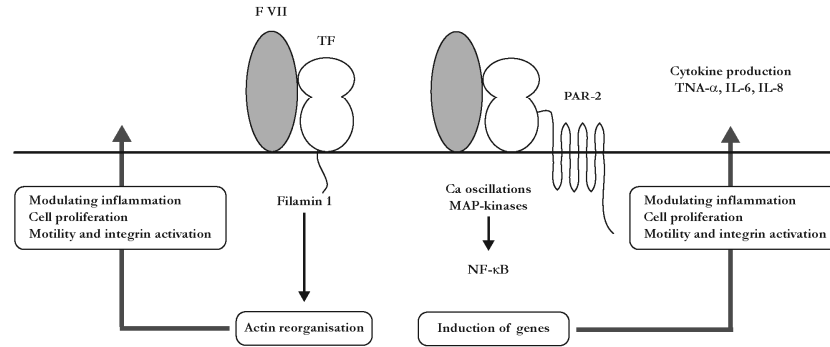
**Figure 6.** The distribution of tissue factor. In the normal setting, TF is not exposed on monocytes, neutrophils and endothelial cells, but this can occur in inflammatory conditions.

The demonstration of circulating, “blood-borne” TF in healthy volunteers, confirms that TF exists not only anchored to cellular membranes (Giesen et al. 1999) (Figure 6). The origin of this circulating form of TF is uncertain. Platelets were initially believed to be the source of blood-borne TF, but this has been contradicted (Butenas et al. 2005, Österud et al. 2008). TF has also been demonstrated in small lipoprotein structures, micro-particles, which may be shed from leukocytes, endothelial cells, vascular smooth muscle cells and platelets (Chou et al. 2004, Mackman 2009). Blood monocytes represent the predominant source of TF in the circulation. Similar to endothelial cells, monocytes constitutively express little TF under basal conditions, but its expression can be enhanced by specific stimuli including C-reactive protein and TNF- $\alpha$  (Breitenstein et al. 2010).

Whether neutrophils express TF upon stimulation or not is still a matter of debate (Österud et al. 2004 and 2010, Nakamura et al. 2004). Although no TF-dependent procoagulant activity has been detected in whole blood stimulated with different agents, it is suggested that neutrophils may be able to express TF under specific inflammatory conditions (Maugeri et al. 2006). The role of microparticle-associated TF is still unclear, but the TF activity in the microparticles seems to be weak, measured by the ability to induce formation of thrombin, (Österud et al. 2008). Yet another form of TF is the circulating, alternatively spliced TF (asTF), lacking transmembrane and intracytoplasmic tail. AsTF has been demonstrated in lungs, placenta and plasma (Bogdanov et al. 2003), and in various cancer cells (Yu et al. 2004). The 165–166 lysine doublet involved in TF–FVIIa interaction has been shown to be intact (Kelley et al. 1995), but the role of asTF is still unclear. One may speculate that asTF binds circulating FVII and thereby acts as an inhibitor of coagulation, by limiting the amount of free FVII able to bind to full-length TF.

#### *Tissue factor-factor VIIa as a signalling receptor*

Apart from its pro-coagulant role, the TF-FVIIa complex has a role as a signalling receptor. Tissue factor resembles a group of pro-inflammatory cytokine receptors and is able to activate a variety of pro-inflammatory intracellular signal transduction routes. TF-FVIIa seems to participate in cell signalling by two distinct mechanisms: proteolysis-independent signalling via the cytoplasmic domain of TF, and proteolysis-dependent signalling, which is independent of TF’s cytoplasmic tail (Siegbahn 2000 and 2005). In proteolysis-independent signalling, filamin 1 is recruited to TF upon its ligation, and this probably provides an essential intracellular link in transmitting signals from TF. Filamin-1 is an actin-cross-linking phosphoprotein that transduces ligand–receptor binding into actin reorganization, which is required for locomotion of many cell types (Pendurthi et al. 2002). In proteolysis-dependent signalling, the TF/FVIIa complex activates protease-activated receptors (PARs), which couple to G proteins, to impact multiple signalling pathways (Siegbahn 2000, Pendurthi et al. 2002) (Figure 7).



**Figure 7.** Tissue factor-FVIIa signalling by two distinct mechanisms: proteolysis independent and proteolysis dependent (via PAR-2)

The PAR family has at least four members (PAR 1-4) (Coughlin 2000), where TF-FVIIa has been shown to be able to act through PAR-2, while TF-FVIIa-FXa also activates PAR-1. PAR-2 is the only PAR not activated by thrombin. PAR-2 regulates TF cytoplasmic tail phosphorylation and downstream gene expression, cell proliferation and survival, as well as motility and integrin activation (Ahamed et al. 2004, Versteeg 2006). Reciprocally, phosphorylation of the cytoplasmic tail of TF also regulates PAR-2 function (Belting et al. et al. 2004). Through PAR-2, TF-FVIIa may activate three major mitogen-activated proteases (MAP)-kinases, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, signalling through downstream activation of transcription factors such as NFκB, AP-1 or EGR-1, which in turn activate other intracellular pathways (Poulson et al. 1998, Liu et al. 2006).

The important role of TF-dependent signalling in regulation of gene transcription, apoptosis and cytoskeleton reformation has been demonstrated in many studies (Watanabe et al. 1999, Abe et al. 1999, Daubie et al. 2007). The role of TF-FVIIa signalling in inflammatory conditions is confirmed by TF-FVIIa regulated expression of the pro-inflammatory cytokine IL-8 in keratinocytes (Wang et al. 2002), and a role in the regulation of both IL-6 and IL-8 expression in monocytes/macrophages (Muth et al. 2005). Confirming the effect of FVIIa on expression of interleukins, recombinant FVIIa administered to healthy humans caused a three- to fourfold increase in plasma levels of IL-6 and IL-8 (De Jonge et al. 2003). A role of TF-FVIIa signalling in regulation of inflammatory genes has been demonstrated in lipopolysaccharide (LPS)-stimulated macrophages, where TF-FVIIa signalling activated genes coding for TNF- α, IL-6, and IL-8 (Riewald et al. 2001).

Recent studies have shown that FVIIa can bind specifically to endothelial protein C receptor (EPCR), a known cellular receptor for protein C and activated protein C, on the endothelium. In the normal setting FVII circulates in plasma in a concentration seven-fold lower than protein C, and they both bind to EPCR with the same affinity. The formation of FVIIa-EPCR complexes neither activates coagulation, nor modulates TF-initiated coagulation. However, it may be speculated that FVIIa interaction with EPCR impairs EPCR-dependent protein C activation and activated protein C-mediated cell signalling by competitive inhibition (Rao et al. 2008). This may be of certain interest in inflammatory conditions with decreased circulating levels of protein C

The primary mechanism of regulation of TF activity is through a process of self-association and dissociation termed encryption and decryption (Eilertsen et al. 2004). The encrypted form of TF is inactive, while the decrypted form is the active pro-coagulant configuration. Decryption of TF in response to specific stimuli, such as tissue damage, cytokines or calcium influx, may involve disulfide-bond formation. Recently it has been suggested that coagulant TF and signalling TF may be two different structural entities (Ahamed et al. 2006).

The increased cellular expression of TF in inflammatory conditions, at least concerning mononuclear cells and probably vascular endothelial cells, is elicited by pro-inflammatory cytokines. The expression of TF seems mostly dependent on IL-6, as demonstrated in studies showing that inhibition of IL-6 completely abrogates TF-dependent thrombin generation in experimental endotoxemia, whereas specific inhibition of other pro-inflammatory cytokines had less or no effect (Van der Poll et al. 1994, Levi et al. 1997). In turn, TF modulates the inflammatory response by affecting the production and the release of inflammatory mediators. In severe sepsis, mononuclear cells, stimulated by pro-inflammatory cytokines, express TF, which leads to systemic activation of coagulation (Almdahl et al. 1987, Kälisch et al. 2007). Even in experimental low-dose endotoxemia in healthy subjects, a 125-fold increase in TF mRNA levels in circulating monocytes can be detected (Franco et al. 2000).

### *Fibrinogen*

Fibrinogen is a plasma acute phase protein produced in the liver, with a half life of 3-5 days. It plays a key role in platelet aggregation, the final step in the coagulation cascade, through the formation of fibrin. Fibrinogen is a major determinant of plasma viscosity and erythrocyte aggregation, and is both expressed in the normal setting and inducible during an acute phase reaction. Normal or low levels of fibrinogen in inflammatory disease are associated with fibrinogen consumption (Ganrot 1997).

Studies in animal models and in human disease have demonstrated that extra-vascular fibrinogen that is deposited in tissues is not merely a marker, but a mediator of



inflammatory diseases (Reinhart et al. 2003, Adams et al. 2007). The ability of fibrinogen to participate in the inflammatory response seems to be dependent on its interaction with specific leukocyte integrins (Ugarova et al. 2001, Flick et al. 2004).

## **Modulating inflammation and coagulation**

Multiple substances with different modes of action have been studied with the aim to modulate the inflammatory response in AP. The investigated modes of intervention include blocking inflammatory cytokines and chemokines (Bhatia et al. 2002), inhibition of adhesion molecules (Lundberg et al. 2001), antioxidants (Mohseni Salehi Monfared et al. 2009), induction and inhibition of nitric oxide (Ozturk et al. 2008, Ang et al. 2009), various protease inhibitors (Chen et al. 2007), enteral nutrition (Eckerwall et al. 2006) and immunonutrients (Petrov et al. 2009). Despite the fact that experimental, and for some substances also clinical results have been demonstrated, no specific therapy has yet been able to revolutionise the treatment of patients with AP.

### *Anti-inflammatory effects of anti-coagulation in acute pancreatitis*

Several studies have demonstrated anti-inflammatory effects of inhibition of TF-FVIIa (Taylor et al. 1991, Levi et al. 1994, Creasey et al. 1993, Taylor et al. 1998, Welty-Wolf et al. 2001, Carraway et al. 2003, Olanders et al. 2005), but no other studies, except from the experimental studies included in this thesis, have been published on inhibition of TF-FVIIa in AP.

Concerning anti-inflammatory effects of other anti-coagulants in AP, heparin has been demonstrated to provide a protective effect in mild experimental AP, probably by reduction of leukocyte-endothelium interaction and normalisation of pancreatic microcirculation (Hackert et al. 2004). Low molecular weight heparin (LMWH) improves microcirculation and survival in severe AP in rats (Qiu et al. 2007), and in a recently published study on treatment with LMWH in patients with severe AP, complication rate, mortality and mean hospital stay were reduced in the LMWH-treated group (Lu et al. 2009). Based on results from smaller clinical studies, heparin has been suggested to be of certain value in hypertriglyceridemic AP, where one of the effects of heparin is to decrease triglycerides by stimulating lipoprotein lipase activity (Tsuang et al. 2009). Both heparin and LMWH have in clinical studies failed to show any value in preventing acute post-ERCP pancreatitis (Rabenstein et al. 2004, Barkay et al. 2008).

Focusing on therapeutic effects of natural anti-coagulants in AP, anti-thrombin III (AT III) ameliorated AP in rat models with mild AP, as well as severe AP (Yamaguchi et al. 1997, Hagiwara et al. 2009, Bleeker et al. 1992). AT III inhibits various

proteases in the coagulation cascade, such as thrombin, factors Xa, IXa and XIIa, but also inhibits some non-coagulation proteases such as trypsin (Levi et al. 2008).

Tissue factor pathway inhibitor (TFPI) has been demonstrated to be raised in patients with AP and to be related to severity (Yasuda et al. 2009). In experimental models of sepsis TFPI has shown anti-inflammatory properties. No studies on TFPI in AP have been published.

Activated protein C (APC) has gained a lot of attention in the treatment of severe sepsis, as recombinant APC was demonstrated to improve survival in a mega-trial (Bernard et al. 2001). In three studies on severe AP in rats APC decreased inflammation and improved survival (Alsfasser et al. 2006), improved histology, superinfection rates and inflammatory markers (Yamanel et al. 2005), and ameliorated AP through modulation of MAP-kinase pathways (Chen et al. 2007). In yet another study early APC treatment did not, however, result in any significant improvement in oxidative and inflammatory parameters nor in renal function (Akay et al. 2008).

When considering treatment related to the crosstalk between inflammation and coagulation in AP, it should be mentioned that much effort has been made to investigate the therapeutic value of a platelet activating factor (PAF) antagonist. PAF is an important proinflammatory mediator acting through specific receptors on various cells and its concentration is raised in a number of diseases, including AP. The ubiquity of PAF receptors on the surface of and within normal cells reflects the physiological importance of PAF. The PAF inhibitor Lexipafant® showed promising results in the experimental setting and in a clinical study (Kingsnorth et al. 1995), but in a large multi-centre study lexipafant failed to show any reduction of organ failure or mortality in severe AP (Johnson et al. 2001).

### *FVIIai*

FVIIai is a competitive inhibitor of the TF-FVIIa complex. FVIIai is synthesised by incorporation of chloromethyl ketone D-Phe-L-Phe-L-Arg into the active site of FVIIa. FVIIai possesses at least the same high affinity for TF as FVII (Jang et al. 1995, Sorensen et al. 1997). The resultant inactive TF-FVIIai-complex does not activate the coagulation cascade, or other intracellular activities associated with TF-FVIIa (Kjalke et al. 1998). The half life of FVIIai is similar to that of FVIIa, i.e. 3-6 hours (Erhardtsen et al. 2001).

Powerful anti-inflammatory effects of FVIIai have been demonstrated in a rat model of ischemia-reperfusion (Olanders et al. 2005), as well as in models of *E.coli* sepsis in baboons (Welty-Wolf et al. 2001, Carraway et al. 2003). With these results in mind, it was disappointing that a recently published randomised controlled trial of FVIIai in

mechanically ventilated patients with acute lung injury or acute respiratory distress syndrome, showed no beneficial effect on morbidity or overall outcome. The cohort of patients receiving the highest dose of FVIIa had increased mortality rates compared with placebo-treated patients, and there was a trend of increased risk of serious bleeding with increasing doses (Vincent et al. 2009).

### *N-acetylcysteine*

Pre-treatment with N-acetylcysteine (NAC) in experimental severe AP reduces levels of myeloperoxidase (MPO) in remote organs and prevent the raise in plasma levels of IL-6 (Shi et al. 2005). Treatment with NAC has been shown to increase levels of glutathione. NAC acts as an antioxidant, both directly as a glutathione substitute and indirectly as a precursor for glutathione. Glutathione is an endogenous antioxidant and has a ubiquitous role in many of the body's defences. It also causes vasodilatation by increasing cyclic guanosine monophosphate levels, inhibits platelet aggregation, acts as a sulphhydryl donor to regenerate endothelial-derived relaxing factor, and reduces IL-8 and TNF- $\alpha$  production (Atkinson 2002).

NAC has been proposed as a treatment for several illnesses, such as hepatic failure, acute respiratory distress syndrome, sepsis, myocardial infarction and as a prophylaxis of radiographic contrast nephropathy (Atkinson 2002). There is strong evidence to support the use of NAC for the treatment of paracetamol overdose, while the use of NAC in other conditions is less well documented. One problem with NAC in clinical use is that it is safe and well tolerated when administered orally, but has documented risks with intravenous administration (Dodd et al. 2008).

## **Haemorrhagic events in acute pancreatitis**

When considering anti-coagulant therapies in AP one must have the risk of major bleeding in mind, which is a rare but potentially lethal complication of severe AP (Andersson et al. 2010). Coagulopathy may be associated with severe AP, making the patients more prone to bleeding. In a study on patients undergoing surgery because of severe necrotising AP, non-survivors had significantly lower levels of activity of the natural anticoagulants protein C and AT III, and higher concentrations of D-dimer and PAI-1 than survivors. However, no differences in INR, APTT, fibrinogen or plasminogen were seen (Radenkovic et al. 2004). These results indicate an impaired anti-coagulant function and an increased fibrinolysis, while the coagulation seems to be intact.

Apart from bleeding associated with an imbalance in the coagulation system, haemorrhage in AP can result from local severe inflammation and an enzymatic insult to major pancreatic or peripancreatic vessels, leading to acute rupture or

pseudoaneurysm formation. Other causes of bleeding include erosion of blood vessels in walled-off necrosis, splenic vein thrombosis with left-sided portal hypertension causing oesophagogastric varices, and pancreatic surgery (Flati et al. 2003, Di Paulo et al. 2006, Ammori et al. 1998, Mortelé et al. 2001, Tsiotos et al. 1996, Kriwanek et al. 1999). Bleeding from ruptured vessels may occur into the gastrointestinal tract, the peritoneal cavity or the retroperitoneum. Gastrointestinal haemorrhage may also be attributed to coexisting lesions such as peptic ulceration, stress ulcers, alcoholic gastritis and Mallory-Weiss tears.

Severe bleeding in AP seems to be a rare event, though previous reports lack figures on the exact incidence (Flati et al. 2003, Di Paulo et al. 2006, Ammori et al. 1998).

## **Coagulation parameters as predictors of severity**

Early assessment of severity is a prerequisite for optimal management of patients with AP. A correct prediction of severity is also important for eligibility for entry into clinical trials. Recent clinical studies have suggested a potential role of coagulation variables, such as TF, tissue factor pathway inhibitor (TFPI), AT III and D-dimer, as predictors of severity in AP. Today, the most widely used laboratory parameter to predict severity of AP and development of complications is C-reactive protein (CRP). Several studies have indicated IL-6 as a superior predictor of severity early in the course of the disease, and IL-8 has also been suggested to be of value. A recent meta-analysis on the ability of IL-6 and IL-8 to predict severe AP concludes that these cytokines perform at an acceptable level in predicting severe AP (Aoun et al. 2010).

Data concerning the role of coagulation variables as predictors of severe AP are still sparse. The results from one study on patients with AP, showed that levels of TF were related to the development of pancreatic necrosis in alcoholic severe AP, but no association with overall severity was demonstrated (Sawa et al. 2006).

In another study on 91 patients with AP, D-dimer, pro-thrombin time and fibrinogen were different in the group of AP patients developing organ failure compared to the patients who did not develop organ failure, both at admission and 24 hours later. D-dimer was the best predictive marker of organ failure (Radenkovic et al. 2009).

In one study on 139 patients with AP, the levels of antithrombin III (AT-III), fibrin/fibrinogen degradation products, platelet count, D-dimer, and thrombin-AT-III complex at admission were associated with severity and prognosis of AP. AT-III, fibrin/fibrinogen degradation products, platelet count, D-dimer, and thrombin-AT-III complex at admission showed larger areas under the ROC curves compared to CRP. AT-III was the best predictor of fatal outcome (Maeda et al. 2006).

In a study on 44 patients with AP, levels of TFPI measured at admission were related to severity (Yasuda et al. 2009).



The overall aim was to investigate the crosstalk between inflammation and coagulation in acute pancreatitis, with special reference to the role of coagulation factor VII and tissue factor, and to study severe haemorrhagic complications in association with acute pancreatitis.

Specific aims for the individual studies were:

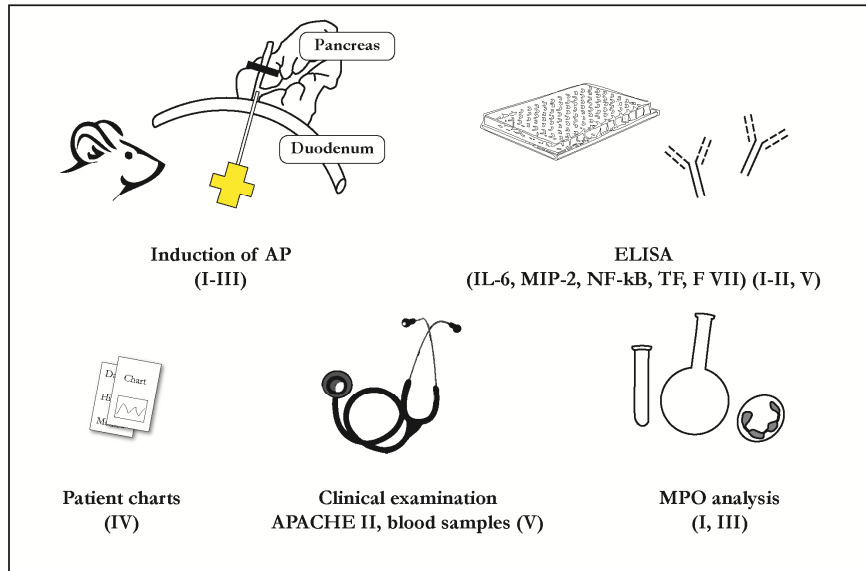
- To investigate the anti-inflammatory effects of active site inactivated factor VII (FVIIai) in experimental severe acute pancreatitis (paper I-III).
- To examine activation of nuclear factor kappa B in severe acute pancreatitis and the effect of FVIIai on nuclear factor kappa B activation (paper II).
- To record incidence, management and outcome of major haemorrhagic complications in severe acute pancreatitis (paper IV).
- To explore plasma levels of tissue factor and factor VII in the early phase of predicted severe acute pancreatitis (paper V).





# MATERIAL AND METHODS

The different methods used are presented in detail in the individual papers, and are only briefly outlined in figure 8. General aspects on material and methods are discussed below.



**Figure 8.** Methods used in the studies. Numbers in parentheses refer to the paper where the individual methods are used.

## *Aspects on experimental severe acute pancreatitis (paper I-III)*

Models of acute pancreatitis established in experimental animals are of four different types: In the duct obstruction models outflow from the pancreatic duct is mechanically inhibited by a surgically placed ligature (Senninger et al. 1984). In the duct injection models bile or bile salts are injected into the pancreatic duct (Aho et al. 1980). In secretagogue-induced models, rodents are injected with the cholecystokinin analogue caerulein in doses exceeding the rate of maximal secretion of pancreatic digestive enzymes. This results in inhibited secretion by yet not fully clarified mechanisms (Lampel et al. 1977, Saluja et al. 2007). In the diet-induced models mice are given a choline-deficient diet supplemented with ethionine. This results in a defect secretion of pancreatic enzymes in response to secretagogue stimulation (Lombardi et al. 1975).

The rat model used in the experimental work of this thesis is of the duct injection-type, and it involves retrograde injection of bile salts into the pancreatic duct (Figure 9). It is a simple, effective and reproducible model for creating a severe, rapidly evolving and lethal variety of acute haemorrhagic pancreatitis, appropriate for studies of systemic effects (Aho et al. 1980). Infusion of 0.2 ml/kg of 3%, 4.5% or 5% taurodeoxycholate (TDC) solution induces acute haemorrhagic pancreatitis with 72-h mortality rates of 24, 71 and 100 percent, respectively (Aho et al. 1980, 1983) (Figure 9). The Sham-operated rats were subjected to laparotomy and identification of the common bile duct, but neither clamping nor any infusion was administered.



**Figure 9.** Model of induction of acute pancreatitis, showing infusion of ink into the rat pancreas. (Photograph reproduced with permission from Jakob Axelsson)

Based on previous pilot studies performed by our group, six hours was chosen as the time point to study. At six hours there will be no mortality, but the rats will be severely ill, with a fulminant form of AP with a SIRS reaction and inflammatory reactions seen in lungs and liver. The gut and renal dysfunction will be more pronounced later in the course of the disease, and mortality will start at about nine hours and then increase until 100 percent after 72 hours (Aho et al. 1980). In the NF- $\kappa$ B study, an early time point, one hour after induction of AP, was added, to detect early changes in NF- $\kappa$ B-activity.

With the TDC-infusion technique the injection pressure with which the solution is applied to cause severe AP must be kept low (Arendt 1993), as any solution, such as normal saline, can produce AP when injected with high pressure into the pancreatic

duct (Condon et al. 1974). Even if the amount of TDC injected and the pressure are the same, the degree of filling of the pancreas may vary between study animals because of inter-individual anatomical differences in the duct system, and this will affect the degree of AP. Another important aspect on this model is that there is limited evidence to support the role of bile reflux in human AP (Elliott et al. 1957). Hence this model may be criticised for not being clinically relevant.

Different animal models of AP has been reviewed elsewhere (Hue Su et al. 2006), and one can conclude that no existing animal model is perfect as an instrument of studying human AP. However, it is important to realise that without animal models there would not be much knowledge on mechanisms involved in early cellular events, pathogenesis and pathophysiology of AP.

#### *Aspects on ELISA (paper I-III, V)*

ELISA is an easy method to use and is considered to be very specific, as antibodies directed against two or more distinct epitopes are required. Nevertheless, several limitations for the interpretation of ELISA data exist. The immunoreactive proteins detected by ELISA may not correlate with the levels of bioactive protein, as an ELISA may utilise antibodies that cannot discriminate between the precursor (inactive) and mature (bioactive) form of a protein. Moreover, an ELISA may detect partially-degraded proteins which have retained their immunoreactive properties (i.e., at least two recognisable epitopes) but may have lost their bioactivity.

As an example, the ELISA used to measure plasma TF recognise both active and inactive forms of TF, and the results do not adequately reflect thrombogenic or pro-inflammatory potential, or micro particle release (Aras et al. 2004). Another uncertainty with this analysis can be low specificity, as it has been proposed that the antibody used in similar ELISA systems is not entirely specific for TF antigen and may cross-react with other proteins (Butenas et al. 2005, Mälarstig et al. 2005).

#### *Aspects on the retrospective chart study (paper IV)*

In retrospective chart reviews, there will always be information that cannot be retrieved. There is no possibility to add clinical parameters that have not been registered, or laboratory analyses that have not been performed. Incomplete documentation increases the risk of missing information, and this is important to consider when interpreting the results of a retrospective study. Considering the clinical challenge of managing patients with severe bleeding it seems plausible to expect that it will be noted in the charts that the patients bleed, at least in the form of intensive care records, multiple haemoglobin analyses, ordering of blood units, angiography results or registration of operations. As the charts of the patients with AP

have been thoroughly investigated concerning such remarks, the risk of omitting patients with AP and severe bleeding seem low in the present study.

*Aspects on the prospective clinical study (paper V)*

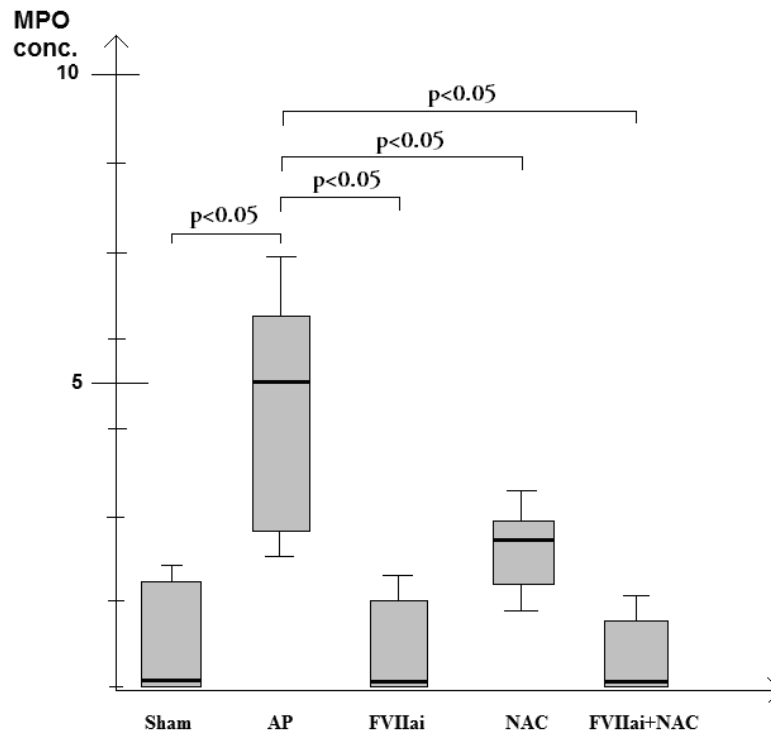
The study was mainly designed to compare the efficacy and safety of early, nasogastric enteral nutrition with total parenteral nutrition in patients with predicted severe AP (Eckerwall et al. 2006), while the focus in the present paper is on tissue factor and factor VII early in the course of severe AP. Data on IL-6 and CRP from this study have already been published (Eckerwall et al. 2006), but not previously analysed with respect to ability to predict severe AP, as done in the present study.

## RESULTS

### *Effects of pre-treatment and treatment with FVIIai in acute pancreatitis, and investigation of NF- $\kappa$ B activation (paper I-III)*

In paper I (Andersson et al. 2007) the results from the model of TDC-induced AP in the rat, showed that the levels of MPO in lungs and ileum, and plasma levels of IL-6 and MIP-2 were higher in the rats with AP, compared to the Sham-operated animals, measured six hours after the induction of AP.

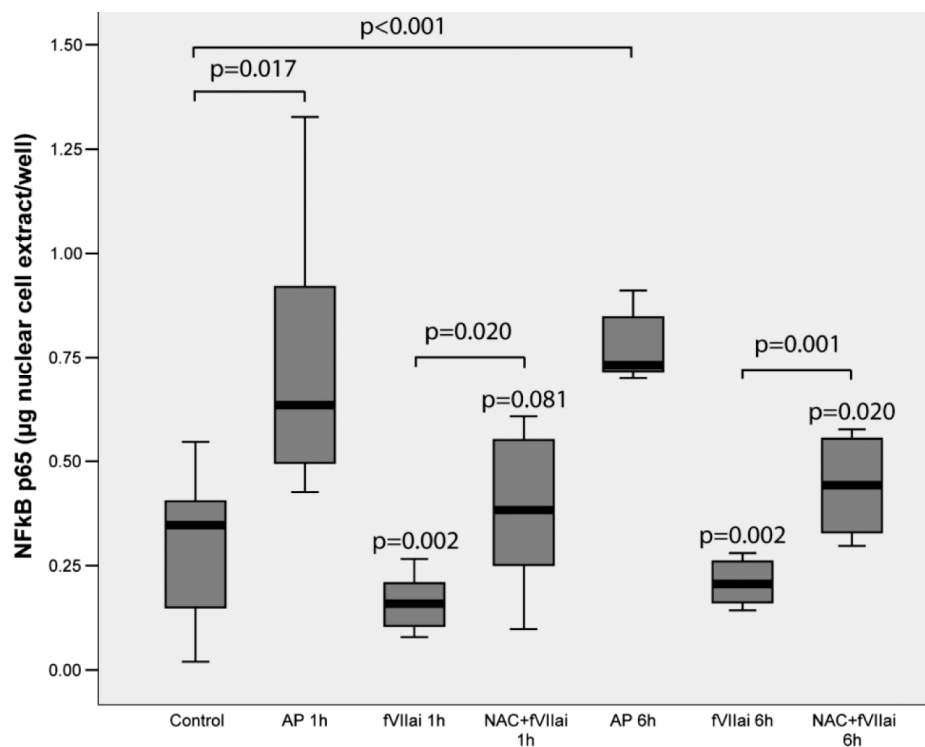
A profound anti-inflammatory effect of active site inactivated factor VII (FVIIai) pre-treatment was demonstrated in the rats with AP, as measured by lower levels of MPO, reflecting a reduced number of activated leukocytes in remote organs (lungs, ileum) (Figure 10) and reduced plasma levels of IL-6 and MIP-2. The anti-inflammatory effect seemed to be at least as pronounced as the effect of N-acetylcysteine (NAC).



**Figure 10.** Pulmonary MPO concentration 6 hrs after induction of AP (Paper I).

In paper II (Axelsson et al. 2008), NF- $\kappa$ B activation in the TDC-induced AP model was investigated. NF- $\kappa$ B was demonstrated to be up-regulated in lungs, liver and ileum. In the liver NF- $\kappa$ B activation was most prominent at the early time point studied (one hour after induction of AP), while NF- $\kappa$ B activation was pronounced both at one and six hours in the lungs. In the ileum NF- $\kappa$ B activation was detected at the later time point (six hours after induction of AP).

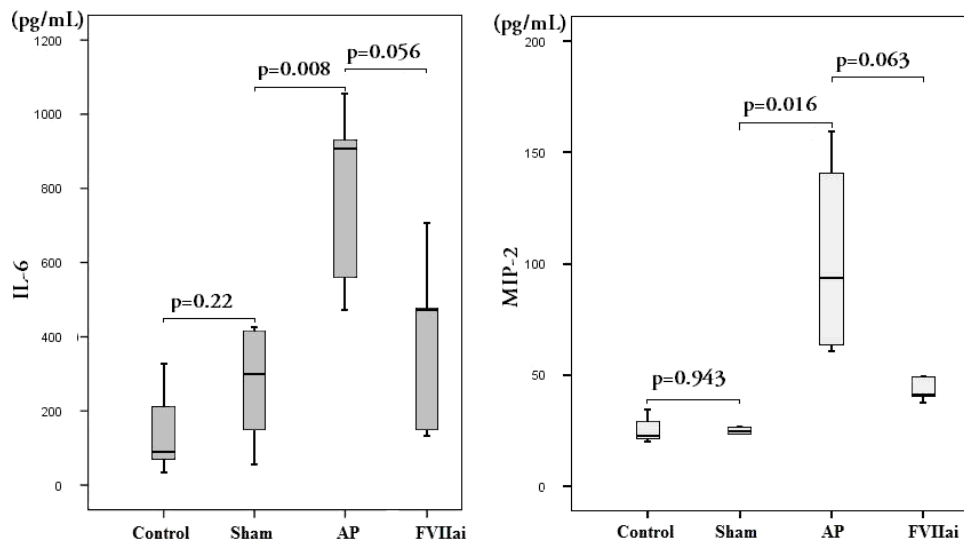
Pre-treatment with FVIIai effectively reduced activation of NF- $\kappa$ B in lungs (Figure 11), liver and ileum. When NAC was given in combination with FVIIai, the anti-inflammatory effect of FVIIai on NF- $\kappa$ B activation was reduced.



**Figure 11.** NF- $\kappa$ B activation in the lungs. Effects both at 1h and 6 hrs (Paper II).

In paper III (Andersson et al. manuscript 2010), FVIIai was administered after the induction of AP, to further investigate the effects of FVIIai in the TDC-induced model of severe AP. The anti-inflammatory effects of FVIIai, when administered 30 min and 90 min after induction of AP, were less evident, as compared to the effects seen with pre-treatment in paper I (Andersson et al. 2007).

When FVIIai was administered 30 minutes after induction of AP, MPO content in the lung was decreased, and there was a tendency towards lower levels of IL-6 and MIP-2 (Figure 12).



**Figure 12.** Concentrations of IL-6 (pg/ml) and MIP-2 (pg/ml) in plasma 6 hrs after induction of AP. FVIIai treatment administered after 30 min (Paper III).

When FVIIai was given 90 minutes after induction of AP, no significant effect of treatment was detected measured by the levels of MPO, but a tendency towards lower IL-6 ( $p=0.055$ ) and MIP-2 ( $p=0.067$ ) levels in plasma were seen in the FVIIai treated animals.

The ileal MPO levels were very low in all of the groups. No statistically significant increase of MPO activity was demonstrated in the ileum in the non-treated AP group, compared to the sham group or to the control group.

It was shown that APTT, and levels of D-dimer and fibrinogen were unaffected, both by acute pancreatitis, and by FVIIai treatment. PT was raised in the rats treated with FVIIai. These findings indicate that the rats with AP in this model do not suffer from DIC or other severe coagulation abnormalities, at least not at six hours after the induction of AP.

*Incidence, management and outcome of major haemorrhagic complications of severe acute pancreatitis (paper IV)*

In this retrospective chart study 1356 patients diagnosed with AP were included. Fourteen patients (1.0 per cent) developed major haemorrhage. Angiography was able to establish a diagnosis in four of six patients and embolisation was successful in one patient. Surgery was performed in two patients. The site of bleeding, management and outcome are summarised in Table 2. Post-operative major bleeding was often preceded by sentinel bleeding; a minor bleeding occurring before the major bleeding. In our study sentinel bleeding was detected in three of four patients with post-operative major bleeding. Overall mortality was high, 5 of 14 patients. Intra-abdominal haemorrhage presenting after >7 days was associated with very high mortality, 4 of 5 patients. Fatal outcome was markedly higher in patients with severe AP and haemorrhagic complications, compared to those with severe AP without bleeding.

**Table 2.** Overview of 14 patients with major haemorrhage in association with acute pancreatitis. GI = gastrointestinal; TAE = transcatheter arterial embolisation; SAP = severe acute pancreatitis; MAP = mild acute pancreatitis; SB = sentinel bleeding (Paper IV).

Site of bleeding	No of patients	Onset of bleeding (day)	SAP/ MAP	Treatment	SB	Fatal outcome
Splenic artery	1	48	SAP	TAE	Yes	1
Gastroduodenal artery	1	9	SAP	TAE	Yes	0
Superior mesenteric artery	1	14	SAP	Surgical ligation	Yes	1
Gastroepiploic artery	1	27	SAP	TAE	No	1
Bleeding ulcer	2	3; 7	MAP	Endoscopic sclerotherapy 1, conservative 1	No	0
Diffuse peripancreatic	3	1; 1; 9	2 SAP, 1 MAP	Conservative 1, Pancreatic. resection 1, Operative drainage 1	No	1
Upper GI	2	1; 1	1 SAP, 1 MAP	Conservative 2	No	1
Lower GI	2	1; 5	2 SAP	Conservative 2	No	0
Unknown	1	1	SAP	Conservative	No	0



*Tissue factor in the early phase of acute pancreatitis and its potential role as a predictor of severe acute pancreatitis (paper V)*

In this prospective clinical study 49 patients with predicted severe AP were included. Inclusion and exclusion criteria are listed in Table 3. Blood samples were collected at inclusion in the study, after 12 hours, after 1 day and after 3 days.

**Table 3.** *Inclusion and exclusion criteria*

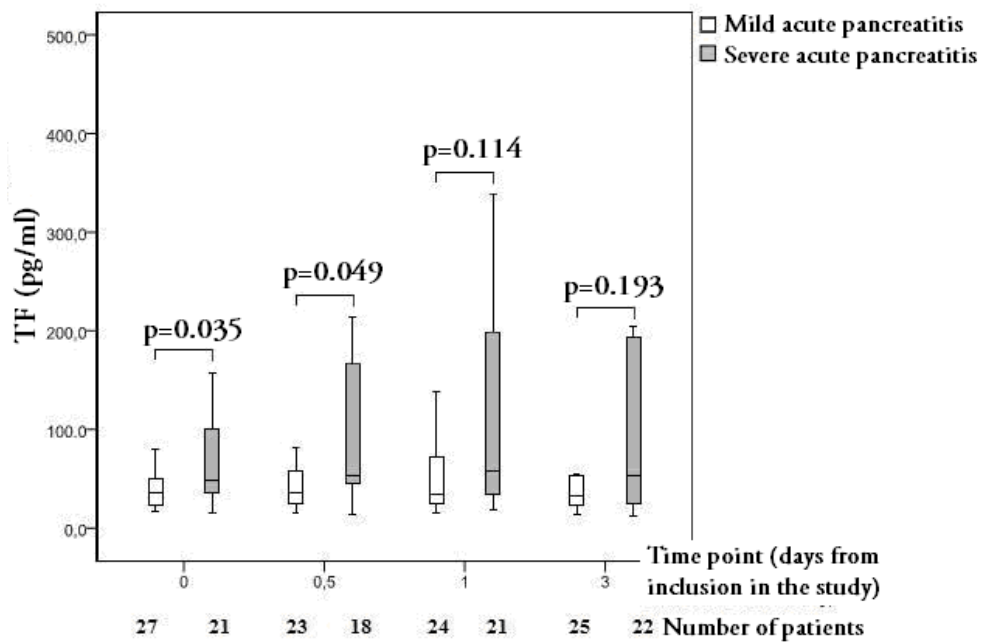
Inclusion Criteria	Exclusion Criteria
> 18 years	Acute pancreatitis due to surgery
Abdominal pain	Trauma
Amylase > 3 times upper normal limit	Cancer
Onset of abdominal pain within 48 hr	Inflammatory bowel disease
APACHE II score > 8 and/or	Stoma
CRP > 150 and/or	Short bowel
Peripancreatic fluid collection on CT	Chronic pancreatitis

According to the Atlanta classification 22 patients (45%) fulfilled the criteria of severe AP and 27 patients (55%) were classified as having mild AP. The severe AP and the mild AP groups were comparable with respect to gender, duration from onset of pain to inclusion and APACHE II score. Median age was lower in the severe AP group (Table 4).

**Table 4.** *Patient characteristics and laboratory variables at time of inclusion. Values are expressed as median and inter-quartile range (Paper V).*

	MAP	SAP	<i>p</i>
Age (years)	76 (63-85)	63 (56-77)	0.042
Sex (male : female)	14:13	10:12	0.66
APACHE II	9 (8-11)	9 (7-13)	0.86
Pain onset to inclusion (hr)	34 (21-43)	25 (22-29)	0.16
Amylase	8.2 (2.7-13.7)	9.8 (4.3-15.3)	0.690
IL-6 (pg/ml)	100 (55-210)	275 (158-315)	0.001
CRP (mg/ml)	106 (69-167)	173 (104-209)	0.
Tissue factor (pg/ml)	35 (23-50)	49 (36-101)	0.035
Fibrinogen (g/l)	4.8 (4.4-6.2)	4.0 (3.8-7.2)	0.047
FVII (ng/ml)	155(46-294)	136 (88-296)	0.989

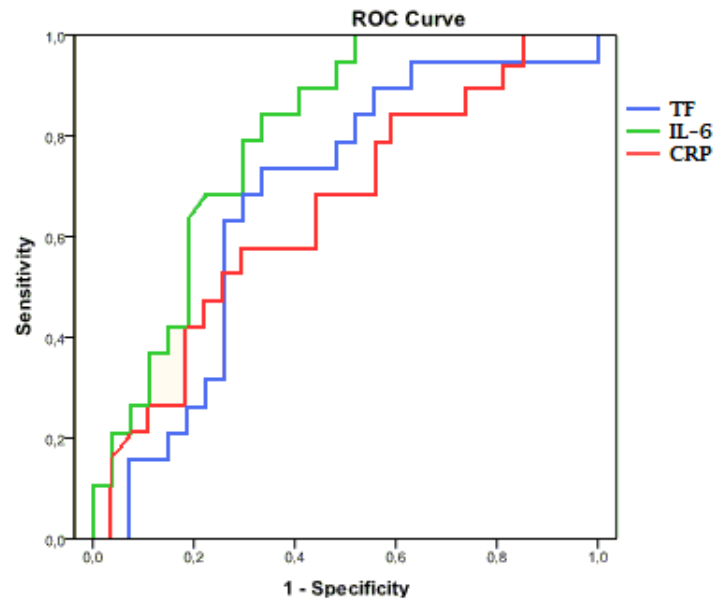
TF was significantly higher in the group of patients with severe AP compared to the mild AP group, both at admission and after 12 hours (Figure 13).



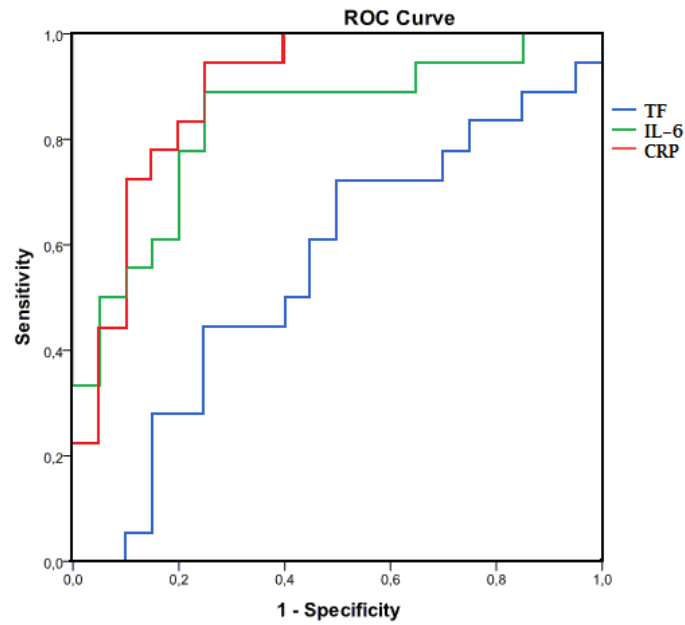
**Figure 13.** Tissue factor in the early phase of acute pancreatitis, 1-3 days after admission (Paper V).

Fibrinogen and FVII were only analysed at admission, and fibrinogen was slightly lower in the severe AP group compared to the mild AP group, whereas no differences were seen in levels of FVII. IL-6 peaked at 12 hours and was higher in the severe AP group at all of the studied time points. CRP peaked at day 3, and was higher in the severe AP group at 1 and 3 days.

Receiver operating characteristics (ROC) curves and area under the curve-analysis were used to investigate the ability to predict severe AP by TF-levels. Different cut-off points for TF were selected based on good sensitivity and specificity, high positive predicted values and low negative predicted values. At inclusion in the study the ability of TF to predict severe AP was inferior to IL-6, but better than CRP (Figure 14). At 12 hours, TF was inferior to IL-6, and at 3 days both IL-6 and CRP were superior to TF (Figure 15).



**Figure 14.** ROC curve of TF, IL-6 and CRP at inclusion in the study (time point 0). AUC TF 0.677,  $p=0.043$ , AUC IL-6 0.775,  $p=0.001$ , AUC CRP 0.653,  $p=0.071$ . (paper V).



**Figure 15.** ROC curve of TF, IL-6 and CRP after three days. AUC TF 0.556,  $p=0.559$ , AUC IL-6 0.833,  $p<0.001$ , AUC CRP 0.892,  $p<0.001$  (paper V).



## DISCUSSION

### *The effect and mechanisms of action of FVIIai in acute pancreatitis*

It may be speculated that the anti-inflammatory effects demonstrated with FVIIai pre-treatment, are induced only by less formation of micro thrombi and resultant reduced micro-vascular dysfunction. However, the absence of coagulopathy in this model of TDC-induced AP, as reflected by unaltered INR, APTT, D-dimer and fibrinogen six hours after induction of AP, supports the hypothesis that it is unlikely that FVIIai exerts its anti-inflammatory effect only by affecting coagulation. Results from other studies on anti-inflammatory effects of FVIIai, confirm this hypothesis, as inhibitors of down-stream coagulation proteases, such as factor X, will result only in inhibition of coagulation, and not in anti-inflammatory effects (Olanders et al. 2005).

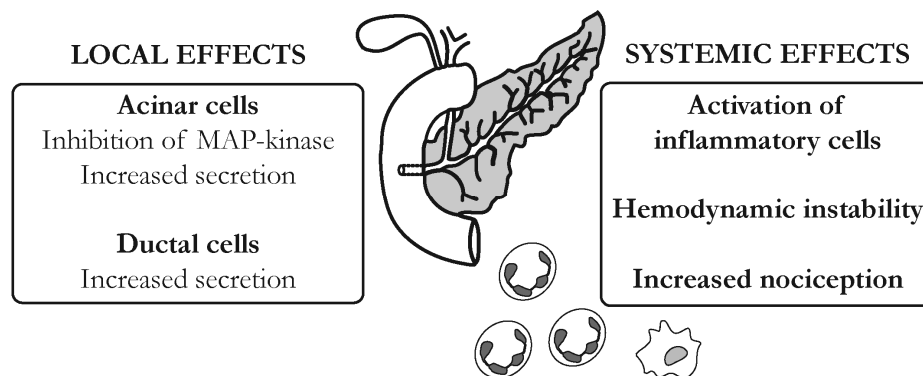
The activation of proteases, which occurs early in AP, allows the activation of protease activated receptors (PARs), considered to be important players in the crosstalk between inflammation and coagulation. Of special interest is PAR-2, activated by TF/FVIIa. The role of PAR-2 in AP may, however, be complex. Stimulation of PAR-2 in the pancreas seems to exert a protective effect by increasing electrolyte and fluid secretion. One can speculate that PAR-2 activation has evolved in the pancreas as a protective mechanism designed to dampen the injurious effects of intra-pancreatic trypsinogen activation (Sharma et al. 2005). Systemic PAR-2 activation may cause hemodynamic instability, activate inflammatory cells and increase nociception, and experimental studies indicate that PAR-2 stimulation may actually worsen severe AP (Laukkarinen et al. 2008).

With this in mind, an explanation of the anti-inflammatory effect of FVIIai seen in our studies may be the reduced systemic stimulation of PAR-2, by the competitive inhibition of the TF/FVIIa PAR-2 stimulation. Although the inhibition of down-stream coagulation by FVIIai can reduce fibrin deposition and micro-thromboses, the decreased NF- $\kappa$ B-activity demonstrated with FVIIai pre-treatment supports the hypothesis that the anti-inflammatory effect is mediated not only by inhibition of the down-stream coagulation cascade, but also by changes in intra-cellular signalling. To make the picture even more complex, recent findings suggest novel anti-inflammatory roles for NF- $\kappa$ B, which means that a systemic reduced activation of NF- $\kappa$ B in inflammatory conditions may be two-edged (Lawrence et al. 2010).

The profound anti-inflammatory effects of pre-treatment with FVIIai, as measured by MPO levels in remote organs, plasma levels of IL-6 and MIP-2, are much less evident when FVIIai is administered 30 min and 90 min, respectively, after the induction of AP. Evidently, FVIIai is being less effective when administered at a more pronounced

stage of the disease. The lack of evident effect in the fulminant stage of the disease may be a reflection of different pathways of the inflammatory response in the early and during the later phase. One explanation may be that the anti-inflammatory effect of FVIIai is mediated predominantly by inhibiting activation of monocytes. This would explain why FVIIai will be efficient only when given as early treatment, when the monocytes are considered to be important actors in the development of the inflammatory response. Another explanation may be the expression of TF and FVIIai. It is known from the clinical situation that plasma concentrations of TF are increased in patients suffering from severe AP (Sawa et al. 2006). This has also been shown experimentally. In rabbits subjected to chenodeoxycholic acid-induced AP, both TF and FVII coagulant activity peak at two hours after infusion (Ottesen et al. 1999). This could explain the loss of anti-inflammatory effects of FVIIai at later stages, by increased amounts of endogenous TF and FVII competing with the inhibitor, FVIIai. The reduction of effectiveness with FVIIai treatment given later in the course of the disease may also reflect the fact that protease activity is an important feature in the early course of AP, whereas other inflammatory mechanisms may be more important later on.

The model of TDC-induced AP results in a fast destruction of the pancreatic tissue, and this model is not suitable when studying local effects in the pancreas. If inhibition of PAR-2 is the mechanism of action of the anti-inflammatory effects of FVIIai in the TDC-induced AP-model, different effects may be seen in models of mild AP, where inhibited PAR-2 stimulation may actually worsen the local inflammation in the pancreas (Figure 16).



**Figure 16.** *Effects of PAR-2 stimulation in acute pancreatitis.*

When presenting the anti-inflammatory effects of FVIIai in our experimental studies, it must be mentioned that the administration of FVIIai has been studied more extensively in sepsis. In a baboon model of sepsis-induced acute lung injury FVIIai

significantly limited the acute lung injury and organ damage. FVIIai improved gas exchange and lung compliance, prevented lung oedema and pulmonary hypertension, and preserved renal function. Treatment also attenuated sepsis-induced fibrinogen depletion and decreased systemic proinflammatory cytokine responses (Welty-Wolf et al. 2001, Carraway et al. 2003). Encouraged by these promising experimental results, a recent clinical trial on FVIIai in subjects with acute lung injury/acute respiratory distress syndrome was performed. FVIIai therapy had no statistically significant treatment effect on coagulation parameters or outcome overall. However, there was a trend towards increased risk of serious bleeding with increasing dose and the highest dose used ( $4 \times 400 \mu\text{g/kg}$ ) was associated with increased mortality, which resulted in early termination of the trial (Vincent et al. 2009).

The ideal treatment would have a high anti-inflammatory effect, while the risk of bleeding should be minimal. In a previous study in our laboratory, FVIIai administered intraperitoneally in rats showed a dose-dependent increase in INR. In the TDC-model the anti-inflammatory effect of FVIIai, as measured by MPO and IL-6, was not stronger when doses of FVIIai were increased above 10 mg/kg (data not published), whereas INR and the haemorrhagic diathesis continued to increase.

### *The clinical relevance of results from studies in rats*

Some issues on the clinical relevance of the TDC-induced AP model have already been addressed in the section on methods. Injection of TDC will result in destruction of the cells in and adjacent to the pancreatic ducts. This fast dissolvment of the pancreatic tissue is not seen in clinical acute pancreatitis, and the systemic effects seen in this model may therefore not be similar to the effects seen in the clinical situation. The problem with uncertain clinical relevance is true for all animal models of AP, and this must be taken into consideration when interpreting the results from animal studies. With studies in cell cultures the situation is not the same as when the cells are in their natural compartment, and if circulating inflammatory cells are retrieved from patients with AP and studied in the lab, they are not in their natural environment.

When studying anti-coagulants in animal models it is important to be aware of the inter-species variation in coagulation. Differences in the concentrations of clotting factors will affect variables used to estimate coagulation, such as INR and APTT, and differences in inflammatory variables must also be taken into consideration. Of certain interest to the experimental studies presented in this thesis is that normal PT-values in rats are slightly higher, but in about the same range as in humans (Siller-Matula et al. 2008). It is of importance to note that rats have a much higher basal level of circulating FVII, and this is also true for FV and FXII, whereas levels of FIX, FX and FXI are lower (Karges et al. 1994). A 100-fold higher dose of thrombin was required in rats as compared to humans, to cause a similar decrease in clotting time (Siller-Matula et al. 2008), and a 20-fold higher dose of a potent FXa inhibitor (DX-

9065a) was required to inhibit thrombus formation (Hara et al. 1995). The pronounced species differences between humans and rats must be kept in mind, when considering the clinical relevance of the anti-inflammatory effect of FVIIai. It is possible that the effect on coagulation and probably also the inflammatory effects of FVIIai in rats may be different from the effects that would be seen in clinical trials of FVIIai.

Despite these discouraging remarks, one must accept that animal models are valuable tools to study mechanisms in the very complex scenario of AP, and though there may be differences, still knowledge with profound clinical impact may be gained from these models.

### *The relevance of results from trials on sepsis*

Results on three different anti-coagulants in mega-trials in sepsis have been presented in the last decade. APC (Bernard et al. 2001) showed a reduction in 28-day mortality, whereas TFPI (Abraham et al. 2003) and antithrombin III (Warren et al. 2001) did not. An intensive debate on the results from these studies has been on-going since the results were presented (Iba et al. 2004, Afshari et al. 2008, Martí-Carvajal et al. 2008), and this matter goes beyond the scope of this thesis. However, it is important to mention that although sepsis and AP have many features in common, such as the systemic inflammatory response, the release of inflammatory and anti-inflammatory cytokines and the development of multiple organ dysfunction, some differences exist (Frossard et al. 2003). When considering extrapolation of results from trials in sepsis these differences are worth consideration.

When comparing the patients suffering from sepsis and AP it is often much easier to determine the onset of disease in AP, where abdominal pain is reported by the patient. This may make it possible to treat the patients in an earlier therapeutic window.

The diagnostic criteria of AP are more specific than in sepsis, possibly making this group of patients more homogenous. The clinical diagnosis of AP is based on the combination of acute upper abdominal pain and elevated plasma levels of pancreas-specific amylase or lipase (Steinberg et al. 1994), while sepsis is defined as a systemic inflammatory response syndrome (SIRS) plus evidence of infection. SIRS is defined by the fulfilment of two or more of the following criteria: Temperature  $>38^{\circ}\text{C}$  or  $<36^{\circ}\text{C}$ , heart rate  $>90$  beats/min, respiratory rate  $>20$ /min or hyperventilation with  $\text{PaCO}_2 <32$  mmHg, white blood cell count  $>12 \times 10^9/\text{l}$  or  $<4 \times 10^9/\text{l}$  (Bone et al. 1992).

Another aspect is that the frequency of underlying disease, such as pulmonary disease and diabetes, may be higher in patients with sepsis. With these differences in mind, it may be worth considering trying therapeutic substances in AP, even though they fail to show any benefit in trials on sepsis.



### *Haemorrhagic complications and the use of anti-coagulation in acute pancreatitis*

The conclusion from the retrospective study (paper IV) is that major haemorrhagic complications in AP are rare, and tend to occur late in the course of the disease. The risk of major bleeding early in the course of the disease seems to be low. Anti-coagulation, such as FVIIai, used early in the early phase of AP, may by inhibitory effects on inflammation, actually decrease the risk of later major bleeding, caused by excessive inflammation and local complications.

Even if a potential treatment with FVIIai in AP is far from clinical application, anti-coagulation in AP is theoretically appealing, both considering the pure anti-coagulant effect potentially reducing micro-thromboses and improving micro-circulation, and considering the potential PAR-2 effect reducing hemodynamic instability and systemic inflammation.

When it comes to the clinical use of anti-coagulants in AP, the use of heparin and low-molecular-weight heparin (LMWH) have attracted interest in AP, as several experimental and smaller clinical studies have suggested that they may prevent post-ERCP pancreatitis. The mechanisms of the protective effects of heparin and LMWH seem to be the reduction of leukocyte-endothelium interaction and the normalisation of pancreatic microcirculation.

Two randomised clinical trials on prophylactic, subcutaneous administration of LMWH or unfractionated heparin respectively, did not show any significant effect on acute post-ERCP pancreatitis (Rabenstein et al. 2004, Barkay et al. 2008). Remarkable results were recently shown by early administration of LMWH in severe AP in a Chinese multi-centre study on 265 patients, where the mortality was much lower in the LMWH-treated group (Lu et al. 2009). Though this study may be criticised on some points, as on the fact that mortality in the control group was unusually high, (>30 per cent), and the rate of patients in the control group subjected to surgery because of pancreatitis was substantial (>11 per cent), the results still remain interesting.

Another aspect on potential benefits of pure anti-coagulant effects in AP are the relatively common CT findings of thromboses in the splenic vein, superior mesenteric artery and portal vein, and splenic infarction in association with AP (Mortelé et al. 2001 and 2004), where anti-coagulation may exert a protective effect.

### *The role of tissue factor in acute pancreatitis*

TF has attracted a lot of interest in studies of interactions between inflammation and coagulation, especially in cardiovascular research. Genetic variations in the TF gene have been associated with clinical outcome in acute coronary syndrome. CRP, the

clinically most frequent used laboratory parameter in evaluation of severity of AP has been shown to stimulate monocytes to produce TF, and TF is considered to be the most important procoagulant substance in rupture of atherosclerotic plaques (Mälarstig et al. 2005).

Plasma TF levels increase in patients with sepsis and acute coronary syndrome, as well as in patients with AP (Sawa et al. 2006). In the study by Sawa, TF levels were related to the development of pancreatic necrosis in alcoholic severe AP, but in contrast with our results no correlation with over-all severity of AP was demonstrated. In our study, the inclusion criteria excluded patients with the mildest form of AP, and only patients with predicted severe AP were included. Yet we were able to demonstrate significantly higher levels of circulating TF in the group that turned out to develop severe AP, according to the Atlanta classification. TF was shown to be elevated early in the course of the disease, implying that if inhibition of TF should be administered it should be started at admission or prophylactic, as in the case of ERCP.

### *The problematic definition of severe acute pancreatitis*

One major problem in the search for predictive markers in AP is to define inclusion criteria for the studies, selecting patients with high risk of developing severe AP. In the TF-study only 45 percent (22/49) of the patients with predicted severe AP included in the study were later classified as actually developing severe AP.

The definition of severe AP may also be problematic. The Atlanta criteria, based on development of local complications and organ failure (Bradley 1993) are widely used. It has been argued that resolution of organ failure within 48 hours suggests a good prognosis, while persistent organ failure is a marker for subsequent death or local complications. Patients presenting with organ failure that resolves within 48 hours, should therefore perhaps not be considered as having severe AP (Johnson et al. 2004).

Studies on the clinical value of parameters of haemostasis in predicting pancreatitis-associated complications are still scarce. Recent studies have demonstrated D-dimer (Radenković et al. 2009) and TFPI (Yasuda et al. 2009) to be correlated to severity of AP. In our study TF is higher in the group of patients with severe AP early in the course of the disease, but our results indicate that the value of TF as an early predictor of severe AP is limited, as IL-6 is superior as a predictive marker at all time points studied (0, 0.5, 1 and 3 days after inclusion in the study). At inclusion in the study, TF seems, however, to be superior to CRP as a predictor of severity.

## CONCLUSIONS

- MPO tissue content and plasma IL-6 and MIP-2 are elevated in severe experimental AP. Pre-treatment with FVIIai effectively reduces the systemic inflammation.
- Active NF- $\kappa$ B is elevated in remote organs in severe experimental AP. Pre-treatment with FVIIai reduces NF- $\kappa$ B activity in an organ and time dependent manner.
- Treatment with FVIIai, i.e. administration of FVIIai after the induction of severe experimental AP, shows some anti-inflammatory effects when administered early in the course of the disease.
- Severe haemorrhagic complications in patients with AP are rare, but clinically challenging and associated with high mortality. Post-operative sentinel bleeding may precede a major bleeding and should be considered a warning sign.
- Plasma levels of tissue factor in the early course of predicted severe AP, are significantly higher in patients who later turn out to actually develop severe AP. The capability of tissue factor to predict severe AP in the early course of the disease is inferior to IL-6, but superior to CRP at admission.



## FUTURE ASPECTS

The anti-inflammatory effects of FVIIai in AP, especially effects on the pancreas itself, would be interesting to evaluate in other models of AP, such as the caerulein-induced model of mild AP in rats. This would elucidate the effect of FVIIai in the pancreas and systemic effects in a model of mild AP. Assuming the role of PAR-2 signalling in FVIIai-action, and remembering the suggested organ-specific effects of PAR-2 stimulation in AP, it is possible that FVIIai would actually worsen the injury in the pancreas itself.

To confirm the suggested PAR-2 effect of FVIIai, PAR-2 knockout mice could be of help. The model of TDC-induced AP in mice could be used to evaluate the effect of FVIIai in PAR-2 <sup>-/-</sup> and wild-type mice, where we hypothesise that the anti-inflammatory effect of FVIIai would be less evident in the PAR-2 knockouts.

The crosstalk between inflammation and coagulation has gained a lot of interest in cardiovascular research. TF is known to be expressed in atherosclerotic plaques, and high levels of circulating TF as well as genetic differences in the TF-gene have been suggested to be linked to an increased risk of myocardial infarction and the risk of ischemic stroke at young age (Sayed et al. 2009 Wannamethee et al. 2009). This may reflect a certain biology in these patients, which could possibly render them more susceptible to other conditions where coagulation and inflammation interact. Our findings of high levels of TF in the early course of severe AP generate questions on whether similar genetic differences exist in patients with severe AP, predisposing them for development of a severe form of the disease. A study of some other genetic haemostatic polymorphisms, including the factor V Leyden mutation, was recently published. No over-representation of such gene polymorphisms were detected in the studied group of patients with severe AP (Tukiainen et al. 2009), but variations in the TF gene have not yet been investigated in AP.

The value of treatment of all AP patients with anti-coagulants early in the course of the disease is controversial. The potential benefits with improved micro-vascular function reducing the pancreatic injury, inhibiting thromboses in the splenic vein and other surrounding vessels, and potential anti-inflammatory properties of anti-coagulants in AP need future investigation. The disappointing results with FVIIai in acute lung injury (Vincent et al. 2009), and TFPI (Abraham et al. 2003) and antithrombin (Warren et al. 2001) in sepsis trials are discouraging, but they do not exclude potential benefits of these substances in AP, and further clinical studies on the effects of anti-coagulation in AP are warranted.

The applicability of coagulation parameters in predicting organ failure and/or severe AP have been suggested in recent studies on D-dimer, antithrombin III, TF and TFPI (Maeda et al. 2006, Yasuda et al. 2009, Radenković et al. 2009). Even though none of these parameters alone seems likely to be of paramount value in predicting severity, these results imply a role of activation of coagulation in the early phase of AP, and the potential role of coagulation parameters as predictors of severity needs further studies.

The complexity of AP makes it plausible to assume that patients with AP present a large spectrum of pathophysiologic events, where one single type of therapy is unlikely to be of benefit for all patients with AP during the entire course of the disease. To be able to reliably predict severity and tailor treatment, a thorough mapping of each individual patient is needed, where coagulation parameters may be one important part.

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## PAPERS I-V

## **Errata**

### **Paper I:**

- It says in the text that MPO in colon was increased in the acute pancreatitis model, and reduced by pre-treatment, however this was not the case. No rise in colon MPO was detected in any of the groups, and hence no effect of pre-treatment was seen in the colon.
- The corrections for multiple comparisons described in the section on *Statistics* has not been taken under consideration in the *Discussion*-part, concerning which results are to be interpreted as statistically significant.

### **Paper II:**

- On page 3 in the section entitled *Statistics*, it should say “ $p < 0.05$  was considered statistically significant”, instead of  $p < 0.005$ .

### **Paper III:**

- Under the third and fourth box-plots presented in the paper, the texts under the pictures are wrong. Under the third box-plot it should say “Figure 3. MIP-2 in plasma (pg/ml). FVIIai treatment administered after 30 min”. Under the fourth box-plot it should say “Figure 4. PT, FVIIai treatment administered after 30 min”.