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BLOOD LEUCOCYTE SIGNALING PROFILES AND TRYPSINOGEN-3 IN SEVERE ACUTE PANCREATITIS

Jani Oiva

ACADEMIC DISSERTATION

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Unigrafia Oy
Helsinki 2017
To my family
ABSTRACT

Acute pancreatitis (AP) is a fairly common disease with a wide range of clinical manifestations. In most cases it is a self-limiting local inflammation. However, about 20-30% of the patients develop a more severe form of the disease (moderately severe or severe AP), which is complicated by systemic inflammation, organ dysfunction (OD) and possibly pancreatic necrosis. In order to prevent mortality, it would be crucial to identify at admission the patients in the risk to develop a more severe form of AP, and to provide them with a maximal supportive care.

The present studies were designed to delineate the blood leucocyte signaling profiles in patients with AP and OD, and to investigate whether circulating trypsinogen-3 is associated with AP.

The study consisted of four parts, in all of which the patients were admitted to hospital because of AP, and treated in the Intensive Care Unit (ICU) for AP complicated by OD and immunosuppression. Studies I-III consisted of a cohort of 13 patients (with additional three patients in study II) and study IV of 82 patients. Phosphorylation of nuclear factor-κB (NFκB), mitogen-activated protein kinases (MAPK) p38 and extracellular signal-regulated kinases 1/2 (ERK1/2), and signal transducers and activators of transcription (STATs) 1,3,5,6 were studied from appropriately stimulated and non-stimulated blood samples using phospho-spesific whole blood flow cytometry. In addition, transmigration of monocytes and polymorphonuclear leucocytes (PMNLs) were investigated using cells of an endothelial cell line EA-HY. Trypsinogen-3 was measured with a new specific sandwich –type immunoassay to investigate it’s clinical utility as a diagnostic and predictive tool in AP.

We found that in AP patients the monocytes have impaired NFκB and STAT1 activation, which may increase susceptibility to secondary infections. p38 activation is normal and STAT3 activation is depressed, which may contribute to the maintenance of systemic inflammation. ERK1/2 activation is impaired which may depress monocytes’ transmigration and may increase the risk for infection.
In AP patients the lymphocytes showed impaired NFκB activation, which may increase the risk for secondary infection. p38 activation is enhanced, which may sustain inflammation. Constitutive STAT3 activation may favour Th17 lineage of CD4+ lymphocyte differentiation. STAT1 activation is impaired and STAT6 activation enhanced, which denotes a shift from Th1 towards Th2 differentiation.

The PMNLs showed depressed NFκB activation, normal p38 activation and decreased ERK1/2 activation. STAT3 was constitutively activated in five patients. Transmigration of the PMNLs was increased, which may sustain end organ dysfunction.

Trypsinogen-3 detected AP patients from controls with high accuracy. However, it did not predict the severity of AP.

In conclusion, these studies show that AP patients with OD and immunosuppression have multiple aberrations in blood leucocyte signaling pathways. These results may offer a potential predictive marker for the development of OD and/or secondary infections. Trypsinogen-3 can detect the patients with AP but does not predict the severity of the disease.
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Acknowledgements

References
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:


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## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANC</td>
<td>acute necrotic collection</td>
</tr>
<tr>
<td>APACHE II</td>
<td>acute physiology and chronic health evaluation II</td>
</tr>
<tr>
<td>AP</td>
<td>acute pancreatitis</td>
</tr>
<tr>
<td>AUC</td>
<td>area under curve</td>
</tr>
<tr>
<td>CARS</td>
<td>compensatory anti-inflammatory response syndrome</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CECT</td>
<td>contrast-enhanced computed tomography</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ECRP</td>
<td>endoscopic retrograde cholangiopancreatography</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formyl-methionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HLA</td>
<td>human leucocyte antigen</td>
</tr>
<tr>
<td>IκB</td>
<td>inhibitor of κB</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>IL-1 receptor antagonist</td>
</tr>
<tr>
<td>ICU</td>
<td>intensive care unit</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus family of protein tyrosine kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MDP</td>
<td>N-acetylmuramyl-alanyl-D-isoglutamine</td>
</tr>
<tr>
<td>NfκB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NOD</td>
<td>nucleotide–binding oligomerization domain</td>
</tr>
<tr>
<td>OD</td>
<td>organ dysfunction</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>arterial partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen–associated molecular pattern</td>
</tr>
<tr>
<td>PCT</td>
<td>procalcitonin</td>
</tr>
</tbody>
</table>
PGN peptidoglycan
PE phycoerythrin
PerCP peridinine-chlorophyll protein
PMA phorbol 12-myristate 13-acetate
PMNL polymorphonuclear leucocyte
PRR pattern recognition receptor
PSTI pancreatic secretory trypsin inhibitor
RFU relative fluorescence units
ROC Receiver operating characteristics
*S. aureus* *Staphylococcus aureus*
SAP severe acute pancreatitis
*S. epidermidis* *Staphylococcus epidermidis*
SIRS systemic inflammatory response syndrome
SOFA sequential organ failure assessment
STAT signal transducer and activator of transcription
Tc cytotoxic T cell
Th helper T-cell
TLR Toll-like receptor
TNF tumor necrosis factor
WON walled of necrosis
1 INTRODUCTION

Acute pancreatitis (AP) is a common cause of acute abdominal pain, with the incidence in Finland as high as 102 cases/100000 inhabitants (Pelli et al 2009). The diagnosis is made of typical clinical symptoms (abdominal pain, nausea, vomiting), with elevated plasma amylase concentration (more than 3 times the upper reference limit) and/or typical findings in contract enhanced computed tomography (CECT). In most cases the etiology is alcohol abuse or gallstones. The clinical course is usually a self-limited disease with mild symptoms and clinical findings. However, up to 20-30% of the patients with AP develop a more severe form of the disease (moderately severe or severe AP), presenting a complication of the pancreas (acute peripancreatic fluid collection, necrosis, pseudocyst) and/or organ dysfunction (OD) (Banks et al 2013. The development of an OD is the major cause for the high morbidity in the patients with AP.

Although much is known about the pathogenesis, the exact mechanism underlying the development of the disease is unknown. For unknown reason premature, trypsinogen becomes activated in the pancreas, leading to autodigestion of the organ. The progress is continued by the activation of local inflammatory cells leading to pancreatic inflammation, i.e. pancreatitis. The body’s inflammatory system tends to restrict this inflammatory process to remain as local one. However, the production of pro-inflammatory mediators may amplify the local inflammatory process resulting in systemic activation of circulating inflammatory cells, known also as systemic inflammatory response syndrome (SIRS). Indeed, the overwhelming SIRS increase the risk of OD and mortality (Mofidi et al 2006).

There is no specific cure for AP. According to the current knowledge the best treatment for patients with AP is supportive fluid therapy, pain relief, early enteral feeding and monitoring (Working Group IAP/APA 2013). Because there is no reliable marker for identifying those patients in a risk of developing a more severe form of the disease, the signs of possible OD should be detected, to predict the possible progression of the disease from mild to moderately severe or severe AP.

Because trypsinogen-3 and blood leucocyte intracellular signaling profiles have not been studied as prognostic markers in SAP, they were investigated in the present thesis in the relationship with outcome of SAP.
2 REVIEW OF THE LITERATURE

2.1 EPIDEMIOLOGY AND ETIOLOGY OF ACUTE PANCREATITIS

AP is a rather common disease and the incidence rate has a vast range of variety between different countries. The lowest incidence rate in Europe has been observed in Albania (5.6/100000 persons per year) (Roberts et al 2017) whereas the highest in Finland (102/100000 persons per year) (Pelli et al 2009). Of the patients with the first attack of AP, up to 17% develop recurrent disease (Ahmed et al 2016).

Several studies show that the incidence rate of AP is rising for unknown reasons (Krishna et al 2017, Roberts et al 2017). This may originate from the increase of alcohol consumption (Pelli et al 2009), the increased incidence of gallstones and obesity (Bonfrate et al 2014). Also, improved diagnostic methods detect milder cases that could have been missed earlier. However, the number of recurrent APs is decreasing (Ahmed et al 2016). The mean or median age at the first attack of AP varies according to the aetiology, from early forties in alcohol – derived pancreatitis to mid-sixties in biliary pancreatitis (Krishna et al 2017, Roberts et al 2017)). The sex distribution in adults is almost equal (Krishna et al 2017).

Despite the increased number of AP, the case fatality rate due to AP has decreased, being as low as 0.79% (Krishna et al 2017). This change could be a result of better diagnostics or better therapy. The highest mortality rate is in patients with AP complicated by infected necrosis and OD (43%) compared with AP complicated by sterile necrosis with OD (22%) and without OD (11%) (Petrov et al 2010). The strongest risk factor for AP-based mortality is advanced age, which may be due to associated diseases (McKay and Imrie 2004).

AP has many different etiologic factors, but the main causes alcohol consumption and gallstones comprises about 80-90% and idiopathic AP 10% of all cases (Tonsi et al 2009). Less commonly, the etiology of AP may originate from metabolic disorders, genetic variations, mechanical obstruction, vascular diseases and autoimmune diseases (Table 1) (Kemppainen and Puolakkainen 2007, Tonsi et al 2009). However, up to 75% of the patients with idiopathic pancreatitis show small (< 3mm) gallstones, which are
poorly detected in conventional transabdominal ultrasound (Smith et al 2015). In Finland, the main etiologic factor is alcohol consumption (Räty et al 2003) comprising about 70% of all cases. In most of the European countries, gallstones are the main etiologic factor of AP (Roberts et al 2017). In recurrent AP, the main cause is alcohol consumption (Pelli et al 2009).

**Table 1. Etiologic factors of acute pancreatitis**

<table>
<thead>
<tr>
<th>Obstructive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholelithiasis</td>
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<tr>
<td>Duodenal diseases</td>
</tr>
<tr>
<td>Pancreas divisum, Sphinkter Oddi dysfunction</td>
</tr>
<tr>
<td>Toxic</td>
</tr>
<tr>
<td>Alcohol</td>
</tr>
<tr>
<td>Drugs</td>
</tr>
<tr>
<td>Endocrine/metabolic</td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
</tr>
<tr>
<td>Hypercalcemia</td>
</tr>
<tr>
<td>Traumatic</td>
</tr>
<tr>
<td>Penetrating and blunt injuries</td>
</tr>
<tr>
<td>ERCP</td>
</tr>
<tr>
<td>Surgery of pancreas/stomach</td>
</tr>
<tr>
<td>Genetic/hereditary</td>
</tr>
<tr>
<td>Genetic mutation of CFTR, PRSS1, or SPINK</td>
</tr>
<tr>
<td>Vascular diseases</td>
</tr>
<tr>
<td>Autoimmune diseases</td>
</tr>
<tr>
<td>Idiopathic</td>
</tr>
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</table>

**2.2 PATHOGENESIS OF ACUTE PANCREATITIS**

Regardless of the etiologic factor, the most common theory of the pathogenesis of AP involves pancreatic acinar cell injury. The early events of AP include reduced exocrine secretion of acinar cells to pancreatic duct, intracellular zymogen activation and the generation of inflammatory mediators (Gaisano and Gorelick 2009). The reduced apical exocytosis of activated zymogens from acinar cells is replaced by sequential basolateral exocytosis to interstitial space (Scheele et al 1987). This together with diffuse proteolysis and pancreatic acinar cell death with the release of activated proteases,
causes damage to adjacent cells and peripancreatic tissue, thus initiating the process of AP.

Although there have been studies on the pathogenesis of AP, the exact initial intracellular mechanism remains open. Most of the theories have been derived from animal studies, for example cerulein-induced acinar hyperstimulation (Ward et al 1996), pancreatic duct ligation (Mooren et al 2003), bile reflux into the pancreas (Kim et al 2002) and alcohol metabolites (Gerasimenko 2009). Common to all these theories is sustained increase of intracellular calcium concentration (Petersen 2008). This leads to intracellular activation of trypsinogens and inflammatory cascades (nuclear factor-κB, NfκB) by unknown pathways and further to the injury of the acinar cell (Gukovsky et al 1998, Dawra et al 2011).

Acinar cell death can occur by at least by two different ways: apoptosis, i.e. programmed cell death or necrosis (Bhatia 2004). Apoptosis is classified in two subgroups, type I and type II, whereas type II is also known as autophagy (Vacaro 2008). Type I apoptosis is characterized by early collapse of cytoskeleton elements and preservation of organelles whereas in autophagy the cytoskeleton is preserved but organelles degrade (Vacaro 2008). Both types of apoptosis develop without plasma membrane breakdown and in the absence of a tissue inflammatory response (Vacaro 2008). The role of autophagy in AP is controversial (Hashimoto et al 2008, Grasso et al 2011). In contrast to apoptosis, necrosis of acinar cell is characterized by the release of intracellular contents into surroundings with local inflammation (Bhatia 2004).

### 2.2.1 Trypsinogens

Trypsins are a family of digestive enzymes that play a significant role in protein digestion. They are synthesized and stored as inactive proenzymes called trypsinogens in pancreatic acinar cells and in cancer cells (e.g. ovarian, gastric, colonic and pancreatic) (Paju and Stenman 2006). Premature activation of trypsinogens in the pancreatic acinar cell for unknown reason is thought to be responsible for the onset of AP.
The pancreas synthesizes three different trypsinogen isoenzymes, i.e. the cationic form (pI 6.2) trypsinogen-1, the anionic form (pI 4.9) trypsinogen-2 and the intermediate form (pI 5.7) trypsinogen-3, which is also called as mesotrypsinogen (Sahin-Tóth 2005). Of these isoenzymes, trypsinogen-1 and -2 forms about 20% of normal pancreatic juice, whereas the proportion of trypsinogen-3 is 3-10% (Sahin-Tóth 2005).

Physiologically these inactive trypsinogens are released into the acinar lumen and further into the duodenum via the pancreatic duct in response to the physiological stimulation of ingested food. After entering to the duodenum, trypsinogens are activated by enteropeptidase (enterokinase) and trypsinogen activation peptide is released (Leung and IP 2006, Petersen 2008).

Over a century ago Hans Chiari proposed that premature activation of trypsinogens inside the acinar cell would be the key point in the pathogenesis of AP. This has been confirmed in an experimental model of transgenic mouse, in which trypsinogen-2 was replaced by endogenously activated trypsinogen (Gaiser et al 2011). There are several theories explaining this activation. First, trypsinogens-1 and -2 have a potential of autoactivation whereas trypsinogen-3 is not autoactivated (Szmola et al 2003). Second, sustained increase of intracellular calcium concentration can lead to premature trypsinogen activation (Shah et al 2009). Thirdly, low extracellular pH sensitizes acinar cells, which predisposes to intracellular trypsinogen activation (Bhoomagoud et al 2009). The major protective mechanism of trypsinogen activation inside the acinar cells is the presence of pancreatic secretory trypsin inhibitor (PSTI) (Pubols et al 1974), also known as SPINK1 (serine, protease inhibitor, Kazal type 1) and tumor –associated trypsin inhibitor (Huhtala et al 1982). PSTI is encoded by the SPINK1 –gene (Pubols et al 1974) and a mutation in SPINK1 –gene is associated with the increased risk of acute and chronic pancreatitis (Tukiainen et al 2005). At physiological concentrations, PSTI is reversibly bound to major trypsinogens thus inhibiting up to 20% of the potential trypsin activity (Pubols et al 1974). However, trypsinogen-3 is naturally resistant to PSTI (Rinderknecht et al 1979) and can degrade it (Szmola et al 2003). Indeed, autophagic colocalisation of zymogen and lysosomal vesicles could lead to cathepsin-B – derived activation of trypsinogen-3, which in turn degrades naturally occurring PSTI resulting in the activated form of trypsin (Halangk et al 2000, Szmola et al 2003).
In the circulation, the PSTI-trypsin–complex is dissociated and active trypsin is inhibited by either $\alpha_2$-macroglobulin or by $\alpha_1$–antitrypsin, also known as $\alpha_1$-proteinase inhibitor. $\alpha_2$-macroglobulin is more inhibitory but because of differences in the plasma concentration (3 µM vs. 26 µM), the $\alpha_1$-proteinase inhibitor plays a more dominant role (Paju and Stenman 2006). Both complexes are rapidly eliminated from circulation by the reticulo-endothelial system.

2.2.2 Inflammation

Inflammation is a finely controlled, protective physiological host response to tissue injury produced by mechanical, chemical or microbial stimuli. The response is mediated by the immune system, which is traditionally divided into two separate systems, innate (non-adaptive) and adaptive. These two systems do not operate separately but instead work concomitantly in concert as part of a complex network of humoral and cellular responses.

All cells involved in the acute inflammatory response originate from pluripotent hematopoietic stem cells, which further give rise to lymphoid and myeloid cell lines. The cells involved in the innate immune system, i.e. monocytes/macrophages, polymorphonuclear leucocytes (PMNLs), eosinophils and basophils/mast cells, are derived from the myeloid cell line whereas the cells involved in the adaptive immune system (T- and B–cells, natural killer cells) originate from the lymphoid cell line.

The innate immune system is activated in response to invading pathogens, which have passed through the first line of defence (i.e. natural barriers of the human body). The cells of the innate immune system recognize pathogens with their non-antigen –specific pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), retinoid-acid-inducible gene-I –receptors, nucleotide-binding oligomerization domain (NOD) –like receptors and lectin –receptors. Their ligands include also endogenous signals released from tissues due to tissue destruction or stress, and microbial components or products, collectively referred to as pathogen-associated molecular patterns (PAMPs). After recognition, the signaling pathway is activated and further, the cells may eliminate the pathogen either by phagosytosis (monocytes/macrophages, PMNLs), or releasing inflammatory mediators, e.g. platelet-activating factor, heparin and prostaglandins into
circulation (mast cells, basophils). In addition, the proinflammatory cytokines, such as TNF-α, IL-6, induce a rapid increase of acute-phase proteins from the liver. These acute phase reactants, including plasminogen, prothrombin, fibrinogen, prekallikrein and C3, play a critical role in activation of other enzyme cascades, such as coagulation, complement, fibrinolysis, kallikrein-kinin –system.

The adaptive immune system, contrary to innate immune system, is highly specific due to its antigen-specific reactions and operates by means of T-lymphocytes (CD3) and B-cells (CD19). T-cells are further divided into CD4 expressing helper T cells (Th) and CD8 expressing cytotoxic T cells (Tc). T-cell activation is mediated by the antigen-presenting cells. Depending on the presented antigen and more importantly, the cytokine secreted by the involved antigen presenting cell, the naive Th-cell (T0) differentiates into Th1, Th2, Th3 or Th17 cell. These cells, as well as activated Tc-cells, have their own distinct functions. B-cells develop into mature plasma cells in response to antigen presentation by Th-cells. Plasma cells are responsible to antigen-specific antibody production, also referred as humoral immune response and immunological memory (Male 2006)

There is increasing evidence that bacteria-induced activation of immune response is mediated by more than one activated pattern recognition receptors. In fact, a proper anti-microbial immune response is mediated by signaling via TLRs and a number of other cell-surface and intracellular PRRs (Trinchieri and Sher 2007).

### 2.2.2.1 Local inflammation

Since the days of Chiari, the “trypsin-centered” –theory of AP has been considered as the triggering event in the process of AP. In addition, recent studies have been defined the role of local inflammation. In experimental pancreatitis, intra-acinar trypsinogen and NfkB are independently activated in the early phase (Hietaranta et al 2001, Ji et al 2009; Jakkambudi et al 2016). However, the local inflammatory process in the development of AP is not related to the trypsinogen activation (Dawra et al 2011). The intra-acinar activation of NfkB as a triggering factor in the development of local inflammation in AP has been discussed for over a decade (Gukovsky et al 1998, Jakkambudi et al 2016). Rapid intra-acinar activation of NfkB has been demonstrated in response to different
stimuli meant to induce AP in experimental studies, but the exact activation cascade of NfκB–activation remains unclear (Rakonczay et al 2008). NfκB induces the expression of proinflammatory mediators including TNF-α from acinar cells (Gukovskayja et al 1997). This is in accordance with findings by Ramudo et al (Ramudo et al 2005), where increased TNF-α expressions were found in the very early phase of AP, contributing to acinar cell of origin. In addition, elevated acinar cell–origin activation of NfκB is correlated to higher severity of AP, through increased content of cytokines (Huang H et al 2013). However, the main source of pro-inflammatory cytokines in the pancreas is macrophages and neutrophils (Norman et al 1995). These may be activated due to early activation of acinar NfκB.

The increased content of proinflammatory cytokines together with increased expression of platelet activating factor results in activation of endothelium and recruitment of circulating leucocytes to the inflamed area (chemotaxis) (Sandoval et al 1996). Proteolytic enzymes and free oxygen radicals released by activated phagocytes causes more cell damage. In addition, activated neutrophils cause the acinar cell to undergo necrosis rather than apoptosis, thus increasing the content of proinflammatory mediators promoting systemic inflammation (Sandoval et al 1996, Ramudo et al 2005). This is in accordance with the finding of Bhatia et al, where the typical cell death is necrosis in severe acute pancreatitis (SAP) and in mild oedematous AP apoptosis (Bhatia 2004). Also the decreased pancreatic microcirculation induced by the local inflammation in the very early phase of AP may predispose tissue ischemia and finally lead to necrosis of the pancreatic cells (Foitzik et al 2002).

2.2.2 Systemic inflammation

In some cases, local pancreatic inflammation may progress to an uncontrolled level, where excessive production of proinflammatory cytokines and activated leucocytes leak into the systemic circulation either via the portal vein or the thoracic duct (Montravels et al 1995). In the liver, hepatocytes and Küpfer cells amplify the systemic inflammation by producing more proinflammatory cytokines, in particular TNF-α and IL-6, which promote synthesis of C-reactive protein (CRP) and procalcitonin (PCT) in hepatocytes (Castell et al 1989). In response to circulating proinflammatory cytokines, endothelial
cells of distant organs become activated enabling circulating leucocytes to migrate into tissues (diapedesis). Also endothelial permeability increases resulting in excess extracellular fluid and edema.

The whole response of this disseminated inflammatory process is defined as systemic inflammatory syndrome response, i.e. SIRS (Bone 1992). Briefly, responses can be observed in molecular components (cytokines, plasma cascades, acute phase proteins) and in cellular components (leucocytes, endothelium) (de Jong et al 2010). SIRS is characterised when two or more of the criteria presented in Table 2 are present.

**Table 2. Criteria for SIRS (Bone 1992):**
**If two or more of the criteria are met the patient has SIRS**

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body temperature</td>
<td>&gt;38°C or &lt; 36°C</td>
</tr>
<tr>
<td>Heart rate</td>
<td>&gt; 90 beats per minute</td>
</tr>
<tr>
<td>Respiratory rate</td>
<td>&gt; 20 breaths per minute OR PaCO&lt;sub&gt;2&lt;/sub&gt; &lt; 32 mmHg or 4.3 kPa</td>
</tr>
<tr>
<td>Leucocyte count</td>
<td>&gt;12x10&lt;sup&gt;9&lt;/sup&gt;/L or &lt; 4x10&lt;sup&gt;9&lt;/sup&gt;/L, or &gt;10% immature forms</td>
</tr>
</tbody>
</table>

Abbreviations:
PaCO<sub>2</sub>, arterial partial pressure of carbon dioxide; SIRS, systemic inflammatory response syndrome

SIRS can develop not only in AP, but also in sepsis, major burns, major surgery and trauma (Bone 1992). Indeed, the SIRS criteria are extremely sensitive and thereby also nonspecific. As the systemic inflammation proceeds, it may result in OD. Briefly, it requires activation of endothelial cells, accumulation of activated leucocytes, fluid sequestration in the extracellular space, (tissue oedema, hypovolemia), decreased microcirculation (i.e. alterations in coagulation system and blood viscosity), proteolytic enzymes and free oxygen radicals released by activated neutrophils and monocytes.

While inflammatory cells are expressing proinflammatory cytokines, concomitant expression of anti-inflammatory cytokines is observed. This is considered to be a normal inflammatory system’s response to restrict SIRS. The phenomenon is termed as
compensatory anti-inflammatory response syndrome (CARS) (Bone et al 1997). In patients with activated inflammatory system, both SIRS and CARS balance the equilibrium of the response. If the restrictive effect is sufficient, the inflammatory reaction dampens and the patient recovers. When the anti-inflammatory response is inadequate, the net effect is pro-inflammatory which may lead to OD with increased risk of morbidity. In addition, the anti-inflammatory response may become excessive, leading to immunosuppression (or immunoparalysis) with increased risk of secondary infections. Usually secondary infections are seen in the late phase of AP (after first week) (Banks et al 2013).

High plasma concentrations of soluble TNF-α -receptor, IL-10 and IL-1 receptor antagonist (IL-1Ra) are present in patients with SAP (Hynninen et al 1999, Mentula et al 2005, Jamdar et al 2006). IL-10 is considered the most important anti-inflammatory cytokine with a capacity of downregulating pro-inflammatory cytokines and decreasing monocyte human leucocyte antigen –DR (HLA-DR) expression (Opal and DePalo 2000, Fumeaux and Pugin 2002). Monocytes with impaired HLA-DR expression show weakened capacity of antigen presentation (Wolk et al 2000). In addition, AP patients with decreased monocyte HLA-DR expression have increased risk for poor outcome (Mentula et al 2003).

In summary, CARS should be considered as an adaptive process to overwhelming inflammatory process, which may lead to anergy of inflammatory cells, followed with immunosuppression and secondary infections in patients with AP (Mentula et al 2004).

As a summary of systemic inflammation, each cell responds to presented cytokine stimulus via highly sophisticated cytokine/receptor –pathway through intracellular signalling cascades with intense cross-talk between them.

### 2.2.2.3 Triggering factors

Cytokines, endogenic triggering factors (Table 3), are low-molecular weight (16-25 kDa) soluble proteins or glycoproteins produced mainly by white blood cells in response to stress, injury or invasive pathogens. The expression of cytokines is very tightly regulated and is modulated by transcription factors. Cytokines cause their effects via specific cell-
surface receptors. The effects of cytokines depend on the cytokine and the target cell. Many cytokines have similar effects explaining the weak benefit of single-cytokine antagonism therapy (Jonhson et al. 2001). Depending on their functions, they are considered proinflammatory (TNF-α, IL-1β, IL-6, IL-8, IL-18, IL-33) or anti-inflammatory ones (IL-1ra, IL-10), whereas some of them have both pro- and anti-inflammatory effects (IL-6, IL-22) (Huan et al. 2016, Manohar et al. 2017).

Chemokines, i.e. chemotactic cytokines, are low-molecular weight proteins produced by a variety of cell types in response to proinflammatory cytokines. Their main functions are attracting inflammatory cells, i.e. monocytes, macrophages and neutrophils, to inflamed area and activating them (Adams and Lloyd 1997). There are four known subfamilies of chemokines, where monocyte chemoattractant protein-1 (MCP-1) is a known chemotactic cytokine for monocytes (Ajuebor et al. 1998). Recent data suggest that cytokines, such as IL-8 and hepatocyte growth factor, serve as severity markers of AP (Nieminen A et al. 2014).

Bacteria, exogenic triggering factors (Table 3), or more specifically, parts of their outer cell wall (lipopolysaccharide [LPS] and muramyl dipeptide [MDP]) and some of their products (e.g. phenyl-methionyl-leucyl-alanine [fMLP]) serve as ligands for the cells of innate immunity thus activating the inflammatory response, including the expression of cytokines.

### Table 3. Triggering factors of inflammation in acute pancreatitis

<table>
<thead>
<tr>
<th>Endogenic triggers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines</td>
</tr>
<tr>
<td>Chemokines</td>
</tr>
<tr>
<td>Proinflammatory cytokines</td>
</tr>
<tr>
<td>Anti-inflammatory cytokines</td>
</tr>
<tr>
<td>Exogenic triggers</td>
</tr>
<tr>
<td>Bacteria</td>
</tr>
<tr>
<td>Structural components</td>
</tr>
</tbody>
</table>

*Endogenic triggers*

**TNF-α** is an early phase pro-inflammatory cytokine, which is converted from a 26-kDa transmembrane protein by TNF-α converting enzyme to its 17-kDa active form.
It has two receptors (TNF–receptor–I and TNF–receptor–II). The former is constitutively expressed in almost all mammalian cells, whereas the latter being expressed in the cells of immune system (Engelmann et al 1990). Activation of these receptors leads eventually to activation of NfκB and mitogen activated protein kinase (MAPK) intracellular signaling pathways (Malleo et al 2007).

Target cells can downregulate their responsiveness to TNF-α by shredding the receptors in serum concomitantly with the increase of TNF-α. The cellular effects of TNF-α depend on the target cell but the main functions are vasodilatation, increase of microvascular permeability, activation of leucocytes, synthesis of other cytokines and the expression of cellular adhesion molecules (Malleo et al 2007).

In AP, TNF-α is mainly expressed from pancreas-infiltrated neutrophils (Rinderknecht 1988, Norman et al 1995), but also pancreatic acinar cells produce and release TNF-α (Gukovskaya et al 1997, Jiang et al 2013). Indeed, the early TNF-α expression from injured pancreatic acinar cells may promote local inflammation (Ramudo et al 2005). During the progression of AP, the serum concentration of TNF-α increases markedly, possible due to increased expression from the pancreas or from non-pancreatic organs (Norman et al 1995, Malleo et al 2007, Jakkambudi et al 2016) thus affecting the development of distant end organ dysfunction. The role of TNF-α in acinar cell death is controversial (Malleo et al 2007, Sendler et al 2013). In experimental pancreatitis, the monoclonal IgG1 anti-TNF-α antibody infliximab has been founded to suppress neutrophil infiltration and alleviate the development of acute respiratory distress syndrome (Kambhapati et al 2014).

IL-1β is an early phase proinflammatory cytokine that is synthesized mainly in stimulated monocytes and macrophages as an inactive 31-kDa pro-IL-1β and is converted to its 17 kDa active form by caspase-1/interleukin-1 –converting enzyme (Black et al 1989). Also pancreatic acinar express IL-1β (Norman et al 1994). IL-1β mediates its functions to target cells via IL-1 receptor type I (IL-1R1) (Kilian et al 1986), whereas the function of the receptor is antagonised by naturally occurring IL-1Ra (Arend et al 1985). The blockade of IL-1 receptor elevates the expression of IL-6 and
TNF-α (Fu et al 1997). In experimental studies a recombinant IL-1Ra anakinra has been noticed to reduce the development of acute respiratory distress syndrome by reducing the accumulation of neutrophils in the lungs (Tanaka 1995 et al) and decrease the degree of pancreatic damage (Norman et al 1995). Also increased concentrations of naturally occurring IL-1Ra have been founded in patients with sepsis and SAP associated with the development of multi-organ dysfunction syndrome (MODS) (Hynninen et al 1999). There is some evidence that decreased IL-1Ra expression due to gene polymorphism reduces the progression of SAP (Powell et al 2001).

IL-1β induces the activation of the NfκB and MAPK -signaling pathways (Dunne and O’Neill 2003). It also stimulates both local and systemic responses of inflammation by releasing other cytokines (e.g. IL-2), increasing the permeability of endothelium and recruiting inflammatory cells on the site of inflammation (Kusske et al 1996).

IL-4 is a 15 kDa cytokine produced by stimulated Th2-cells, basophils and mast cells. The functions of IL-4 on target cells are mediated by the IL-4 receptor complex, which is formed by a 140 kDa IL-4Rα -subunit and either the common γ -chain or the IL-13Rα1 – chain, leading to activation of the signal transducer and activator of transcription (STAT)-6 signaling pathway (Nelms et al 1999). IL-4 has pleiotropic effects on inflammatory reactions. It induces the proliferation of T -lymphocytes and modulates the differentiation of Th2 -lymphocytes and expression of immunoglobulins of B-cells (Kaplan et al 1996).

IL-6 is a 26 kDa glycoprotein expressed by various cells, eg. T– cells, B-cells, monocytes and neutrophils (Kishimoto 1989). Also pancreatic acinar cells have been founded to express IL-6 (Kim et al 2000). IL-6 has pleiotropic effects on acute inflammation. It decreases the synthesis of pro-inflammatory cytokines IL-1β and TNF-α while increasing the synthesis of IL-Ra and the release of soluble TNF-α -receptor (Tilg et al 1997, Xing et al 1998). On the other hand, IL-6 stimulates the differentiation of antibody producing cells, macrophages and Th17 -cells (Kishimoto 1989; Chen et al 2007) and induces the production of CRP from hepatocytes (Castell et al 1989). Its functions on
target cells are mediated by the IL-6 receptor, a heterodimer consisting of a specific 80 kDa IL-6Rα -chain and a 130 kDa transmembrane gp130 –chain, the latter allowing the activation of the STAT-1 and STAT-3 –pathways (Stahl et al 1995, Gerhartz et al 1996).

**Granulocyte-macrophage colony-stimulating factor (GM-CSF),** also known as CSF2, is a cytokine secreted from a number of stimulated cells (e.g. endothelial cells, monocytes, macrophages) with both mitogenic and activational properties on granulocyte– and macrophage cell lines. It also features with proinflammatory effects on monocytes and macrophages with the co-expression of another stimulus (e.g. LPS) (Hamilton 2002). The effects of GM-CSF are mediated by GM-CSF -receptor, a heterodimer consisting of binding and signaling subunits, both expressed on the surface of leucocytes. The signaling subunit does not have intrinsic tyrosine kinase activity but tyrosine phosphorylation of the cytoplasmic tail of the signaling subunit in association with the Janus family of protein tyrosine kinase (JAK) -2 leads eventually to the activation of the STAT5 –signaling pathway (Guthridge et al 1998).

**MCP-1,** also known as CC-chemokine ligand 2 is a product of a variety of cells, monocytes/macrophages being the major source (Yoshimura et al 1989). Its functions are mediated by two CC-chemokine ligand 2–receptor isoforms, the major form expressed by mononuclear and vascular smooth muscle cells and the minor form expressed in monocytes and activated natural killer -cells (Charo et al 1994). Activation of the CC-chemokine ligand 2–receptor leads eventually to the activation of intracellular signaling cascades and further to migration of the cell along chemokine gradient.

*Exogenous triggers*

**LPS,** a component of the gram-negative bacteria membrane, is one of the most potent activators of macrophages (Rietschel and Brade 1992). The immune responses of LPS in innate immune cells, i.e. macrophages, neutrophils and dendritic cells, are mediated by TLR4 in complex with LPS binding protein, CD14 and myeloid differentiation-2 –proteins (Medzhitov 2007). TLRs are a family of type I membrane glycoproteins that sense
PAMPs derived from pathogens, acting as PRRs. To date, 10 TLRs have been discovered in humans, and their specific ligands have been largely characterized (Achek et al 2016). CD14 is an adaptor molecule, a cell-surface receptor of lipoprotein binding protein lacking a cytoplasmic domain, which identifies LPS and transfers it to the TLR4-myeloid differentiation-2 -complex. Myeloid differentiation-2 is a soluble protein that associates with TLR4 allowing its dimerization after LPS recognition (Fitzgerald et al 2004). TLR4 mediates the signaling in two different ways through interaction with four different Toll-interleukin-1 receptor domain-containing adaptor proteins thus leading to activation of NFκB and p38 (Kaisho and Akira 2006) Early activation is mediated by TIRAP-MyD88 -proteins in the membrane whereas the late activation is mediated by TRAM- and TRIF -proteins in the endosome (Kagan et al 2008). In bone marrow-derived macrophages, both early and late activation mechanisms are required for sustained NFκB activation and the production of inflammatory cytokines (Jiang et al 2005, Kagan et al 2008).

**MDP** is a breakdown product of a bacterial structure peptidoglycan and is the minimal structure inducing immunologic adjuvant activity. The effects of MDP in target cells are mediated by NOD -like receptors. NOD is a cytolocal protein family of PRRs, which seems to have an important role on intracellular immune defence. Nod1 and Nod2 detect fragments of peptidoglycan (PGN) and Nod2 (caspase recruitment domain) is activated by MDP (Girardin et al 2003). Nod1 is expressed in B-lymphocytes and Nod2 in monocytes, macrophages and in T- and B-lymphocytes (Gutierrez et al 2002, Petterson et al 2011). After recognition, Nod1 and Nod2 activate the NFκB and MAPK pathways.

MDP has direct effects on the immune system, e.g. stimulation of lymphocytes (Souvannavong et al 1990) but most effects is synergistic with LPS (Jørgensen et al 2001) or priming effects (Wang et al 2001).

**Staphylococcus aureus (S. aureus)** and **Staphylococcus epidermidis (S. epidermidis)** are gram-positive bacteria whose outer cell wall is composed of multiple PGN layers and lipoteichoic acids linked to the cytoplasmic membrane but lacking LPS. The immune functions of white blood cells on gram-positive bacteria are mediated by
bacterial lipoproteins through the TLR-2 receptor (Hashimoto et al 2006). The PGN mediated signaling of gram-negative bacteria through TLR-2 receptor is controversial (Dziarski and Gupta 2005). However, PGN signaling may be mediated via Nod2 receptor in conjunction with the TLR-2 receptor, at least in dendritic cells (Volz et al 2010). After recognition, the intracellular NFκB pathway is activated.

**Escherichia coli** (*E. coli*) are gram-negative bacteria, whose outer cell wall is composed of thin layer of PGN, LPS and phospholipids. LPS serves as a ligand for the TLR-4 receptor complex leading eventually to activation of intracellular signaling mechanisms (NFκB).

fMLP is a powerful neutrophil chemotactic tripeptide produced by *E. coli* (Marasco et al 1984). The dose-dependent effects of fMLPs are mediated by two subtypes of receptors present on the surface of neutrophils. The high-affinity receptor mediates the chemotactic response of and the low-affinity receptor activates the MAPK signal transduction pathways, where p38 activation plays a central role in regulating neutrophil chemotaxis (Le et al 2001).

**Phorbol-12-myristate-13-acetate (PMA)**, a surrogate of 1,2-diacylglycerol, is a potent tumor promoter often used in research to activate the signal transduction enzyme protein kinase C, which activates extracellular signal-regulated kinase (ERK) 1/2 signaling pathway (Werry et al 2006).

### 2.2.2.4 Signaling pathways

#### 2.2.2.4.1 NFκB

NFκB is associated with immune and inflammatory reactions, such as acute phase response, in response to stress induced by infection or tissue injury by regulating transcription of the genes involved. It was first identified in 1986 as intronic enhancer of
the kappa light chain (κB site) in B cells (Sen and Baltimore 1986). It also can protect from or induce apoptosis in certain cell lines (Sonenshein 1997). NfkB dysregulation occurs in various diseases, including autoimmunity diseases (e.g. diabetes), cancer (e.g. lymphoma) acute and chronic inflammatory processes (e.g. AP, rheumatoid arthritis)

In mammals, the NfkB transcription family consist of five different proteins, NfkB1 (p105/p50), and NfkB2 (p100/p152), p65 (RelA), RelB and c-Rel, where p105 and p100 are precursors of functional proteins. All five are characterized by the presence of an N-terminal Rel homology domain, which is responsible for hetero- and homodimerization of different NfkB –proteins and interaction with inhibitor of NfkB (IκB) –proteins. Binding of NfkB to the specific κB –site of DNA is mediated by the nuclear localization sequence (Zheng et al 2011). However, DNA binding affinity, sequence specificity and transcriptional activity may be altered by posttranslational modulations of NfkB –proteins (Wan et al 2007). Because the RelA:p50 heterodimer was the first form of NfkB to be identified, the term of NfkB is often used to describe that complex.

In non-stimulated cells, NfkB-dimers are held inactive in the cytoplasm by their inhibitors, IκBs, whereas several cell lines (e.g. non-small cell lung carcinoma) exhibit persistent NfkB-activity (Rayet and Gélinas 1999). IκBs can be divided into classical IκBs and atypical IκB –like proteins (Ghosh and Karin 2002). Although the IκBs share similarities in their structure, they have different binding properties, which are subjects to differential transcription regulation by NfkB itself (Weil et al 1997).

Activation of NfkB –signaling pathway is extremely carefully controlled. The key point is degradation of IκBs by a specific IκB-kinase (Scheidereit 2006). This kinase consists of three subunits, the catalytic α- and β -subunits and the regulatory γ -subunit. Depending on the stimulus, the activation process is mediated by the classical/canonical pathway or alternative/non-canonical pathway. The classical pathway is essential in normal development and function of the immune system, whereas the alternative pathway is important in the regulation of central and peripheral immune tolerance (Brown K et al 2008).

As a summary, degradation of the inhibitory factor of NfkB leads to the release and translocation of active NfkB into the nucleus and by recognizing the specific sequence
motifs (κB site) of target genes, they modulate the transcriptional activation of DNA in genes encoding cytokines, chemokines, adhesion molecules, acute phase proteins, secondary inflammatory enzymes and regulators of apoptosis and cell proliferation (Ghosh and Karin 2002).

The activation of NfκB is normally rapid and transient but constitutive NfκB activity occurs in several diseases (Courtois and Gilmore 2006). The degradation of classical IkBs and activation of NfκB in response to stimulus constitutes a negative feedback loop, in which activation of NfκB is terminated. NfκB–induced IkBs enter the nucleus, where NfκB’s higher affinity to IkBs leads to dissociation from DNA and association with IkBs, and further to exportation of inactivated NfκB to the cytoplasm (Jakkampudi et al 2016). Also other de-activation processes of NfκB’s exist (Lawrence et al 2005).

**Figure 1. NfκB –signaling**

Abbreviations:
IkB, inhibitor of NfκB; IKK, IkB –kinase; LPS, lipopolysaccharide; NfκB, nuclear factor –κB; NIK, NfκB –inducing kinase; Nod2, nucleotide-binding oligomerization domain –2; TNF-α, tumor necrosis factor –α
2.2.2.4.2 MAPK

Mitogen-activated protein kinases (MAPKs), are a family of intracellular protein kinases that play a significant regulatory role in various cellular processes, i.e. proliferation, differentiation, development, cell survival, migration and apoptosis. There are distinct MAPK cascades, such as ERK1/2, c-Jun amino-terminal kinases -1, -2 and -3 and p38 isoforms (Irrera et al 2014).

ERK1 (p44 MAPK3) and ERK2 (p48 MAPK1) share 83% similarity of amino acid identity (Irrera et al 2014). The extent of expressed isoforms in cells is variable, but ERK2 is the predominant form. In general, ERK1 and ERK2 are considered as functionally comparable (Shaul and Seger 2006). Physiologically, ERK1/2 participates in the development of the immune system (i.e. monocytic differentiation, mast cell development), antigen activation (T-cell maturation) and cell cycle control in response to growth factors and hormones (Rubinfield and Seger 2005). On the other hand, disturbances in ERK1/2 regulation pathway have been detected in various cancers, Parkinson’s disease and Alzheimer’s disease (Werry et al 2006).

In resting cells, the components of the ERK signaling cascade are distributed throughout the cytoplasm. The cascade is usually initiated at membrane receptors in response to growth factors, hormones and neurotransmitters. The activation of small G proteins leads to multiple sequential phosphorylation processes leading to dual phosphorylation and activation of native ERK1/2 (Rubinfield and Seger 2005, Wotzel and Seger 2011). The final result of the ERK1/2 signaling cascade depends on the hundreds of potential substrates originating in several cellular components. In the nucleus, the activated forms of ERK1/2 modulate nuclear proteins and transcription factors (Wotzel and Seger 2011).

ERK signaling is dependent of the duration and strength of the signal cascade (Shaul and Seger 2006). The activated signal is faded by ERK activated MAPK phosphatases, also known as dual specificity phosphatases, which dephosphorylases the activational loop of ERKs (Ramos J 2008).

The family of p38 -kinases play important roles in immune and inflammatory responses, regulation of cell survival and differentiation in response to various stimuli (e.g. stress, inflammatory cytokines, hypoxia and ischemia). The family consists of four
members, p38α, -β, -γ and δ and they share 62-75% identity to each other. However, they have different expression, activation and substrate specificity resulting from their aberrant functions in different cellular milieus. The predominant form, p38α (MAPK14), is expressed in significant levels in most cells, whereas p38β (MAPK11) is expressed mainly in brain, p38γ (MAPK12/ERK6) in skeletal muscle and p38δ (MAPK13) in endocrine glands. Also, several alternatively spliced variants of p38α exist (Cuadrado and Nebreta 2010). In general, due to the ubiquitous expression of p38α in different cell lines, the term p38 is used to refer to p38α.

Activation of p38 is mediated in response to multiple factors, including inflammatory cytokines (TNF-α, IL-1) and bacterial products (e.g. LPS). The canonical activation pathway of p38 MAPKs is triggered in response to various stimuli (Dong et al 2002). This leads to a series of sequential phosphorylations with activation of MAPK kinases (Cuadrado and Nebreta 2010). The alternative activation pathway is activated in response to T-cell receptor stimulation in T-lymphocytes, leading to autostimulation of p38α kinase.

In resting cells, p38 isoforms are present both in the cytoplasm and the nucleus where they have numerous (>200) potential substrates to phosphorylate, including protein kinases, cytosolic proteins and transcriptional factors. The major effects of p38 activation are the production of proinflammatory cytokines, differentiation of inflammatory cells and proliferation and survival of the cells (Dong et al 2002).

The activation of p38 –kinases is terminated by phosphatases, which are, in response to stimuli, transcriptionally up-regulated and dephosphorylate activated p38 –kinases (Cuadrado and Nebreta 2010).
Abbreviations:
ERK, extracellular signal-regulated kinase; JNK, c-Jun amino-terminal kinase; MAPK, mitogen–activated protein kinase; MAPK2 = M KK, mitogen–activated protein kinase-kinase; MAP3K, mitogen–activated protein kinase-kinase-kinase; MEK, MAPK/ERK–kinase

2.2.2.4.3 STATs

STATs were identified first by Fu et al during the study of gene induction by interferons (Fu et al 1992). STATs literally transduce the signal from the activated receptor to the nucleus and activate gene transcription.

At least seven different STAT-proteins (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) are known (O’Shea et al 2008). Of these, STAT1, STAT3, STAT5a and STAT5b are activated in response to several distinct cytokines, whereas only a few cytokines activate STAT2, STAT4 and STAT6 (Lim and Cao 2006).

The activation mechanism of STATs is receptor-related. At least five different mechanisms are known, i.e. the JAK-STAT pathway, direct activation, non-receptor tyrosine kinase activation, G-protein coupled receptor activation and JAK–adaptor protein activation (Lim and Cao 2006). Of these, the JAK-STAT activation pathway is referred to as the classical pathway. JAK is a cytoplasmic kinase that is activated in
response to activation of cytokine receptor and leads eventually to the phosphorylation of STAT-protein (pSTAT). In mammals, four different JAK –kinases (JAK1, JAK2, JAK3 and Tyk2) are known. The JAK-STAT –signaling pathway is activated in response to several cytokines, including interferons and interleukins, growth factors and some hormones (O'Shea et al 2008).

Despite the activation mechanism, after activation, pSTATs form homo/heterodimers, leave the receptor and translocate into the nucleus. In the nucleus, phosphorylated STAT-dimers bind to specific DNA-sequence to activate gene transcription. Also unphosphorylated STATs are able to form either homo- or heterodimers and translocate to and from the nucleus in association with nucleoporins. Unphosphorylated STATs are also transcriptionally active but the gene expression differs from that of activated forms of STATs (Cheon et al 2011).

Constitutive STAT –activation occurs with STAT-1, STAT-3 and STAT-5 and is associated with various cancers (e.g. breast cancer, esophageal cancer, pediatric acute lymphoblastic leukemia, pancreatic cancer) and wound healing (Lim and Cao 2006).

Activated STAT –dimers typically induce genes synthesizing interferon –inducible synthetases (STAT1), acute-phase –proteins α-2-macroglobulin and fibrinogen (STAT3) and cell-cycle regulator Cyclin D1 and anti-apoptotic Bcl-XL (STAT5) (Lim and Cao 2006). Furthermore, activated STAT1 and STAT3 induce the expression of the genes their own (Yang et al 2005). The genes induced by unphosphorylated STATs are associated with antiviral and immune responses and cancers (Cheon et al 2011).

STATs also induce the transcription of STAT inhibitors, suppressor of cytokine signaling –proteins, also known as cytokine-inducible SH2-containig protein, JAK-binding protein and STAT-induced STAT inhibitor. Currently, there are at least eight different suppressor of cytokine signaling proteins. They inhibit STATs by suppressing JAKs or by competing for binding of the receptor. Other inactivation mechanisms are direct interaction by constitutively expressed protein inhibitor of activated STAT and phosphatation and degradation of STATs (Lim and Cao 2006).
**Cross-talk between signaling pathways**

The regulation of the immune system cells is mediated through different cytokines and hormones. However, certain cytokines may activate different receptors and depending on the involved receptors, activate different signaling pathways simultaneously. These signaling pathways may influence each other, a phenomenon known as cross-talk. For example, induction of IL-6 gene expression by canonical NfκB activation in response to IL-1 or TNF leads to STAT3 expression and thus, increased unphosphorylated STAT3 – levels sustain NfκB –dependent activation of gene transcription.

**2.3 CLINICAL MANIFESTATIONS AND CLASSIFICATION**

The main clinical symptoms of AP include epigastric pain, nausea, vomiting, fever and tachycardia. In some rare cases of SAP (3% of patients), ecchymoses of the flank (Grey-
Turner’s sign) and in periumbilical region (Cullen’s sign) may appear, and are associated with a 37% rate of mortality (Meyers et al 1989). However, there are no specific clinical sign of AP. Indeed, AP can present in a painless patient. On the contrary, the incidence of AP in the patients with sudden death is 0.4% (Tsokos and Braun 2007).

Since September 1992, AP has been classified in terms of widely accepted Atlanta classification (Bradley 1993), which divides AP into mild and severe form of the disease. However, the classification has been widely criticized and finally, a new version was published on 2012 (Banks et al 2013). This update defines the diagnostic methods, imaging findings, types and severity of AP. Contrary to the previous classification the current classification discriminates two time phases of AP due to two different peaks of mortality, i.e. the early phase (lasts usually for the first week), and late phase (lasts from week to months) (Banks et al 2013). These are discussed more detailed in chapter 2.4.3.2 Systemic complications.

**2.3.1 Types of acute pancreatitis**

*Interstitial oedematous pancreatitis*

The majority (80-90%) of the patients with AP are presented by interstitial oedematous pancreatitis, which is described by enlargement of the pancreas due to inflammatory oedema with homogenous enhancement in CECT. The patients are treated by the means of conservative treatment and the mortality rate is very low. The clinical symptoms usually resolve within one week after onset (Banks et al 2013).

*Necrotizing pancreatitis*

The necrotizing pancreatitis (5-10% of AP patients) is presented with necrosis of the pancreas and/or peripancreatic tissues, described as non-enhanced tissue in CECT due to impaired perfusion. The development of necrosis takes few days to evolve and therefore necrotizing pancreatitis as a term should be used earliest if seen after first week (Banks et al 2013).
**Infected pancreatic necrosis**

Pancreatic or peripancreatic infection develops in 32% of patients with sterile necrosis (van Santvoort et al 2011). The infection of the necrotic area during the first week is rare (Banks et al 2013). The infections are often monomicrobial (60-87% of cases) but can occur as polymicrobial, consisting of both aerobic and anaerobic bacteria (13-40% of cases), or even fungi (21% of cases) (Bakoyiannis et al 2010). There are several theories for the origin of the micro-organisms, i.e. haematologous route, transmural migration through the intestinal wall or via ascites, the biliary duct and pancreatic duct and the lymphatics (Bakoyiannis et al 2010). The most typical bacteria are gram - negative aerobic bacteria, comprising 56% of all bacterial cultures from infected necrotic pancreas (Tsui et al 2009). The majority of infections are detected within 14 to 26 days after admission (Brown et al 2014), resulting in increased hospital stay and mortality compared to non-infected patients (van Santvoort et al 2011).

Acute renal dysfunction and immunosuppression increases the risk of infection of the pancreas to 38% (Bakoyiannis et al 2010, Li et al 2010). However, there is no correlation between the extent of necrosis and the risk of infection and the duration of symptoms (Banks et al 2013). Microcirculatory dysfunction in the colonic mucosa may weaken the mucosal barrier, thus potentiating bacterial translocation and infection (Foitzik et al 2002).

The signs of secondary infection of the necrotic pancreas are usually detected by CECT with a presence of gas within the necrotic collection. A positive culture or gram –stain assessed by fine needle aspiration confirms the diagnosis Working Group IAP/APA 2013).

2.3.2 Severity of acute pancreatitis

Compared to the previous classification, the current classification categorizes AP into three categories: mild, moderately severe and severe AP (Banks et al 2013) (Table 4) instead of two (mild and severe AP) (Bradley 1993) (Table 4).
### Table 4. Classifications for severity of acute pancreatitis

<table>
<thead>
<tr>
<th>Classification</th>
<th>Mild AP</th>
<th>Severe AP</th>
<th>Moderate AP</th>
<th>Persistent ≥ 48h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Atlanta 1992 (Bradley 1993)</strong></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local complications</td>
<td>-</td>
<td>+</td>
<td>And/Or</td>
<td></td>
</tr>
<tr>
<td><strong>Organ Dysfunction</strong></td>
<td>-</td>
<td>+</td>
<td>And/Or</td>
<td></td>
</tr>
<tr>
<td>APACHE II ≥ 8 or Ranson ≥ 3</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Classification</th>
<th>Mild AP</th>
<th>Moderately severe AP</th>
<th>Severe AP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Atlanta 2012 (Banks et al 2013)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local complications</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Co-morbidity</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Organ Dysfunction</strong></td>
<td>-</td>
<td>Transient ≤ 48h</td>
<td>Persistent ≥ 48h</td>
</tr>
</tbody>
</table>

Abbreviations:
AP, acute pancreatitis; APACHE II, Acute Physiology and Chronic Health Evaluation II
1) acute fluid collection, pancreatic necrosis, pseudocyst
2) shock (systolic blood pressure < 90 mmHg), pulmonary insufficiency (PaO₂ ≤ 60 mmHg), renal failure (serum creatinine level ≥ 177 μmol/l after rehydration) and gastrointestinal bleeding (> 500 ml in 24 hours)
3) acute peripancreatic fluid collection, acute necrotic collection, walled-off necrosis, pseudocyst
4) exacerbation of pre-existing co-morbidity
5) Modified Marshall Score ≥ 2

### 2.3.3 Complications of acute pancreatitis

#### 2.3.3.1 Local complications

The previous classification described, local complication as pancreatic necrosis, pseudocyst and abscess (Bradley 1992), while in the current classification a local pancreatic complication is described as acute peripancreatic fluid collection, acute necrotic collection (ANC), walled of necrosis (WON) and pancreatic pseudocyst. Other non-pancreatic local complications are gastric outlet dysfunction, splenic and portal vein thrombosis and (Banks 2013).

An acute peripancreatic fluid collection is best described as a wall-less collection of homogenous fluid in the retroperitoneal space that develops from interstitial edematous pancreatitis usually in the early phase of AP. These sterile collections tend to solve spontaneously but may develop into a pseudocyst with the time (Banks 2013).
The current classification divides necrotizing pancreatitis into ANC and WON. The main difference between these manifestations of necrotizing pancreatitis is that the heterogenous collection of fluid and necrotic tissue is surrounded by well-defined inflammatory wall in WON, but a wall is present in ANC. In addition, the development of ANC takes usually less than four weeks after onset, whereas WON occurs after four weeks (Banks 2013).

Pancreatic pseudocyst is characterized as a sterile peri-pancreatic fluid collection persisting more than 4 weeks after onset as a consequence of disrupted pancreatic duct. It is defined by a collection of pancreatic juice enclosed by a well-defined wall of fibrous or granulation tissue (Banks et al 2013). Pseudocysts occur as a complication of AP in less than 10% of patients (Cui et al 2014).

The development of a local complication during the early phase is rare. Therefore radiological examinations should be postponed to run within 72-96 h after onset of symptoms, if necessary (Working Group IAP/APA 2013).

2.3.3.2 Systemic complications

**Organ dysfunction**

Clinical characteristics of the patients with OD induced by AP are analogous to those induced by severe burn, sepsis or multiple trauma (Wilson et al 1998). The activation of inflammatory system during the early phase of AP in response to pancreatic injury may either resolve, may develop to SIRS or may be followed by CARS (Bone et al 1997, Johnson and Abu-Hilal 2004). It is the host’s response to inflammatory reactions that determines the outcome. The dynamic nature of SAP accounts for two-peaked mortality. Indeed, SIRS presenting in the early phase of AP increases the risk of developing OD with high mortality. In the current classification OD is defined as transient, if it resolves within 48 hours and persistent, when OD persist more than 48 hours (Banks et al 2013). In the late phase, the development of CARS or persistent SIRS in patients with SAP increases the probability of infected pancreatic necrosis and OD, both accompanied with a high mortality up to 43% (Petrov et al 2010). In addition, local complications develop during the late phase.
In patients with AP, the importance of early detection of developing a more severe AP is significant. Indeed, due to improved modern intensive care, the rate of early deaths (within 14 days of onset) as a result of OD has decreased, and the most of deaths occur after 14 days due to secondary infection of the necrotic pancreas and OD (Bumbasirevic et al 2009).

The current classification defines dysfunction of three different organ systems, i.e. respiratory, cardiovascular and renal (Banks 2013). Also exacerbation of pre-existing co-morbid disease is defined as a systemic complication (Banks et al 2013). The previous classification defined four different ODs (gastrointestinal bleeding in addition) (Bradley 1992). Other organs, such as liver, coagulation system and immune system, can develop OD as a consequence of AP as well (Halonen et al 2002).

OD can be quantified by scoring systems designed for use in intensive care units (ICU), i.e. multiple organ dysfunction score (Modified Marshall Score) (Table 5) (Marshall et al 1995) and sequential organ failure assessment (SOFA) (Table 6) (Vincent et al 1996), the latter being more accurate in evaluating cardiovascular dysfunction (Bota et al 2002). In the current classification the presence of an OD is determined by modified Marshall Scoring system (Table 5.).

Pulmonary complications are the most predominant OD in patients with AP, comprising 75% of cases (Browne and Pitchumoni 2006). Of the patients with AP, 15-20% develop acute respiratory distress syndrome, the worst complication of the pulmonary system (Browne and Pitchumoni 2006).

Acute renal dysfunction is a common complication in patients with AP, with the incidence rate of 14-42%. Age, hypoxemia, acute compartment syndrome and preceding renal insufficiency are reported to be risk factors for acute renal failure in patients with AP (Li et al 2010).

More than 60% of the patients with SAP have intra-abdominal hypertension (de Waele and Leppäniemi 2009). Untreated intra-abdominal hypertension may lead to inadequate abdominal arterial perfusion pressure and further, development acute abdominal compartment syndrome with the association of new OD.
Multiple organ dysfunction syndrome as a term was defined by the conference consensus by the American College of Chest Physicians and the Society of Critical Care Medicine (ACCP-SCCM) to characterize critically ill patients with altered organ function such that homeostasis cannot be maintained without intervention (Bone 1992).

OD is present in 40% of patients with AP (Petrov et al 2010) and up to 51% of them have OD at admission (Johnson and Abu-Hilal 2004). Patients with persistent (≥ 48 hours) OD have a greater risk of death than those with transient (resolving within 48 hours) OD (Johnson and Abu-Hilal 2004). The mortality rate is associated with the injured organ: 43% in respiratory dysfunction, 55% in cardiovascular dysfunction, 56% in coagulation dysfunction, 81% in renal dysfunction and 83% in liver dysfunction (Halonen et al 2002). Also, the total number of affected organs increases the mortality rate, from 18% (one affected organ) to 69% (four to six affected organs), where the combination of hepatic and renal dysfunctions had the highest mortality (91%) (Halonen et al 2002).

**Extrapancreatic infections**

Also extrapancreatic infections are common in patients with AP, the incidence being 32%. The most common extrapancreatic infections are respiratory infection (9.2%), bacteremia (8.4%) and urinary tract infection (2.6%). The patients with infected pancreatic necrosis are more likely to have extrapancreatic infection. Extrapancreatic infections occur at the late phase of AP. However, some of them may be as hospital-acquired infections, including catheter-associated urinary tract infection, catheter-associated blood stream infection and ventilator-associated pneumonia. Distinguishing hospital-acquired infection from primary infection is controversial. Extrapancreatic infections increase the hospital stay and mortality, despite the origin (Brown et al 2014).

### 2.4 Diagnostic Methods

Making a right diagnose for a patient with AP is crucial because those patients need close hospital surveillance and care. The diagnosis of AP is made on the basis of a
pattern of clinical examinations, laboratory tests and radiology. The most used laboratory test for AP is plasma amylase concentration. In AP, amylase concentration in plasma elevates rapidly, within 12 hours after onset, but decreases to normal within three to five days (Clavien et al 1989). Increased plasma amylase concentration is not very sensitive for AP. Indeed, 19% of the patients with AP have normal plasma amylase concentration (Smith et al 2005) and of the patients with alcohol-induced AP, 32% show normal plasma amylase concentration (Spechler et al 1983). Using the cut-off level more than three times of normal upper limit increases the specificity for amylase in detecting AP (Steinberg et al 1985). Elevated concentrations of urine amylase can be detected in patients with AP and a urine amylase strip test has been developed for a screening test in AP (Hedstöm et al 1998).

Serum lipase is also been used as a diagnostic tool for AP. Its better detection in AP compared to that of serum amylase is thought to derive from its slower elimination (Frank and Gottlieb 1999). Of the patients with AP, only 3% have normal serum lipase concentration (Smith et al 2005). The routine use for either serum amylase or lipase or both is pointless unless there is a suspicion of AP (Sutton et al 2009).

Elevated concentrations of trypsinogen-2, measured either from plasma (Itkonen et al 1990) or from urine (Hesdtröm et al 1996), have been detected in patients with AP. A rapid urine trypsinogen-2 dipstick test for detecting AP is available (Kemppainen et al 1997).

Also trypsinogen-2 derivatives (trypsinogen activation peptide, trypsin-2-α1-antitrypsin) have been studied as a diagnostic tool for AP (Hedström et al 1994).

The value of CECT in detecting AP has been known for decades. However, the findings in CECT can be normal especially in the mild forms of AP thus representing a sensitivity of 91% (London et al 1989). Early CECT should be limited to those patients with an uncertain diagnosis, to confirm severity due to clinical findings, or to those patients with poor response to conservative treatment or clinical deterioration (Working Group IAP/APA 2013).

Taken together, to assess the diagnosis of AP requires symptomatic patient with elevated serum amylase/lipase concentrations more than three times of upper reference limit and/or typical findings on CECT (Banks et al 2013).
2.5 SEVERITY ASSESSMENT OF ACUTE PANCREATITIS

The lack of prognostic markers that identify, on admission to hospital, the patients at the risk for developing moderately severe or severe AP is at present a major challenge to a clinician. Clinical examination on admission is unreliable in assessing the subsequent course AP. Therefore a reliable and clinically useful method to identify patients with developing SAP would be crucial. To achieve this, plenty of different scoring systems, laboratory tests and radiological examinations have been developed.

2.5.1 Scoring systems

Several multi-factorial scoring systems have been developed to assess the severity of AP. Of these, the Ranson (Ranson et al 1974) (Table 8) and Glasgow/Imrie (Blamey et al 1984) –scores are deficient because of the time delay of 48 hours after onset. The acute physiology and chronic health evaluation (APACHE) II (Table 7) score shows similar predictivity to the Ranson score (Knaus et al 1985). Although the use of the APACHE II –score is not time-dependent, it is too sophisticated system for daily clinical use.

Also tests developed to detect OD, i.e. the multiple organ dysfunction score (Modified Marshall Score) (Table 5) (Marshall et al 1995) and SOFA (Table 6) (Vincent et al 1998) can be used in severity assessment but not as an early predictive test.

In addition, a new prognostic scoring system has been developed. The BISAP (bedside index for severity of acute pancreatitis) –score is determined within 24 hours after onset by five parameters: blood urea nitrogen more than 25 mg/dL, impaired mental status, SIRS –score, age over 60 and pleural effusions (Wu et al 2008). It is clinically feasible to use, repeatable and less expensive. When compared to other scoring systems, it shows a predictive value similar to that of APACHE-II and Ranson –score (Papachristou et al 2010).

Taken together, the different scoring systems, although complex to use in clinical practice, may be helpful in detecting the patients more likely to develop a moderately severe or severe form of AP.

<table>
<thead>
<tr>
<th>Organ system</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory (PaO₂/FiO₂)</td>
<td>&gt;400</td>
<td>301-400</td>
<td>201-300</td>
<td>101-200</td>
<td>≤101</td>
</tr>
<tr>
<td>Renal (serum creatinine, μmol/l)</td>
<td>134</td>
<td>134-169</td>
<td>170-310</td>
<td>311-439</td>
<td>&gt;439</td>
</tr>
<tr>
<td>Cardiovascular (systolic blood pressure, mmHg)</td>
<td>&gt;90</td>
<td>&lt;90, fluid response</td>
<td>&lt;90, no fluid response</td>
<td>&lt;90, pH&lt;7.3</td>
<td>&lt;90, pH&lt;7.2</td>
</tr>
</tbody>
</table>

Abbreviations:
PaO₂, arterial partial pressure of oxygen; FiO₂, fraction of inspired oxygen

Table 6 SOFA Score (Vincent et al 1996)

<table>
<thead>
<tr>
<th>SOFA score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiration PaO₂/FiO₂ (mmHg)</td>
<td>&gt;400</td>
<td>≤400</td>
<td>≤300</td>
<td>with respiratory support ≤ 200</td>
<td>≤ 100</td>
</tr>
<tr>
<td>Coagulation platelets (x10³/ml)</td>
<td>&gt;150</td>
<td>≤150</td>
<td>≤100</td>
<td>≤50</td>
<td>≤20</td>
</tr>
<tr>
<td>Liver function bilirubin (mg/ml)</td>
<td>&lt;20</td>
<td>20-32</td>
<td>33-101</td>
<td>102-204</td>
<td>&gt;205</td>
</tr>
<tr>
<td>Cardiovascular hypotension no MAP&lt;70mmHg dopamine ≤ 5 or dobutamine (any dose) dopamine &gt; 5 or epinephrine ≤ 0.1 or nor-epinephrine ≤ 0.1 dopamine &gt; 15 or epinephrine &gt; 0.1 or nor-epinephrine &gt;0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNS GCS</td>
<td>15</td>
<td>13-14</td>
<td>10-12</td>
<td>6-9</td>
<td>&lt;6</td>
</tr>
<tr>
<td>Renal creatinine (μmol/l) or urine output &lt;110</td>
<td>110-170</td>
<td>171-299</td>
<td>300-400</td>
<td>&gt;440</td>
<td>200ml/day</td>
</tr>
</tbody>
</table>

Abbreviations: PaO₂, arterial partial pressure of oxygen; FiO₂, fraction of inspired oxygen; MAP, mean arterial pressure; CNS, central nervous system; GCS, Glasgow Coma Scale
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>0-4</td>
</tr>
<tr>
<td>Mean arterial pressure</td>
<td>0-4</td>
</tr>
<tr>
<td>Heart rate</td>
<td>0-4</td>
</tr>
<tr>
<td>Respiratory rate</td>
<td>0-4</td>
</tr>
<tr>
<td>PaO₂</td>
<td>0-4</td>
</tr>
<tr>
<td>Serum Na⁺</td>
<td>0-4</td>
</tr>
<tr>
<td>Serum K⁺</td>
<td>0-4</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>0-8</td>
</tr>
<tr>
<td>Hematokrit</td>
<td>0-4</td>
</tr>
<tr>
<td>White Blood Count</td>
<td>0-4</td>
</tr>
<tr>
<td>Glasgow Coma Scale</td>
<td>0-15</td>
</tr>
<tr>
<td>Age</td>
<td>0-6</td>
</tr>
<tr>
<td>Chronic health problems</td>
<td>0-5</td>
</tr>
<tr>
<td>(Liver cirrhosis, NYHA IV,</td>
<td></td>
</tr>
<tr>
<td>Severe COPD, regular</td>
<td></td>
</tr>
<tr>
<td>dialysis, immunocompromized)</td>
<td></td>
</tr>
<tr>
<td>Total points:</td>
<td>0-73</td>
</tr>
</tbody>
</table>

Abbreviations:
PaO₂, arterial partial pressure of oxygen;
NYHA IV, New York Heart Association class IV;
COPD, Chronic obstructive pulmonary disease
Table 8 Ranson Criteria for Severity of Acute Pancreatitis (Ranson et al 1974)

<table>
<thead>
<tr>
<th>At Admission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
</tr>
<tr>
<td>White blood count</td>
</tr>
<tr>
<td>Blood glucose</td>
</tr>
<tr>
<td>Serum LDH</td>
</tr>
<tr>
<td>Aspartate transaminase</td>
</tr>
<tr>
<td>During Initial 48 hours</td>
</tr>
<tr>
<td>Hematocrit decrease</td>
</tr>
<tr>
<td>Blood urea nitrogen increase</td>
</tr>
<tr>
<td>PaO₂</td>
</tr>
<tr>
<td>Base deficit</td>
</tr>
<tr>
<td>Serum calcium</td>
</tr>
<tr>
<td>Fluid sequestration</td>
</tr>
</tbody>
</table>

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>&gt; 55 years</td>
</tr>
<tr>
<td>White blood count</td>
<td>&gt; 16.0 x 10⁹/l</td>
</tr>
<tr>
<td>Blood glucose</td>
<td>&gt; 11 mmol/l</td>
</tr>
<tr>
<td>Serum LDH</td>
<td>&gt; 350 IU/l</td>
</tr>
<tr>
<td>Aspartate transaminase</td>
<td>&gt; 250 U/l</td>
</tr>
<tr>
<td>Hematocrit decrease</td>
<td>&gt; 10%</td>
</tr>
<tr>
<td>Blood urea nitrogen increase</td>
<td>&gt; 1.8 mmol/l as urea</td>
</tr>
<tr>
<td>PaO₂</td>
<td>&lt; 60 mmHg</td>
</tr>
<tr>
<td>Base deficit</td>
<td>&gt; 4 mEq/l</td>
</tr>
<tr>
<td>Serum calcium</td>
<td>&lt; 2.0 mmol/l</td>
</tr>
<tr>
<td>Fluid sequestration</td>
<td>&gt; 6 l</td>
</tr>
</tbody>
</table>

Abbreviations: LDH, lactate dehydrogenase; PaO₂, arterial partial pressure of oxygen;

2.5.2 Laboratory tests

An enormous effort has been used in searching for a laboratory test to detect patients with an elevated risk for SAP. The ideal one would be simple to measure, reliable, accurate and repeatable.

In terms of primary pancreatic insult, pancreatic enzymes and their derivatives have been detected to show predictability in SAP. These include urinary –trypsinogen activation peptide (Gudgeon et al 1990), plasma trypsinogen-2 (Sainio et al 1996), plasma trypsin-2-α1-antitrypsin –complex (Hedström et al 1996) and carboxypeptidase-B activation peptide (Appelros et al 2001).

In terms of AP as an inflammatory process, the range of different markers is wide. The most widely used marker in inflammatory processes is CRP. High CRP –levels have been detected in patients with SAP (Puolakkainen et al 1987). Although considered as an acute phase protein, the peak concentration of CRP is reached only 48-72 hours after onset. CRP is therefore unsatisfactory in assessing severity in the early phase but is
applicable within 48 hours after onset (Mofidi et al 2006). Another acute phase protein, amyloid-A, shows almost the same predictivity as CRP (Mayer et al 2002).

Because the inflammatory process of developing SAP is ongoing, the most useful and accurate markers should be inflammatory cytokines. Indeed, high concentrations of IL-1β (Mayer et al 2000) and IL-18 (Rau et al 2001) may predict the development of SAP. The concentrations of serum TNF-α and its soluble receptors have been used as prognostic factors in SAP (De Beaux et al 1996) but TNF-α’s low half-life in plasma (14-18 min) worsens its clinical relevance whereas the half-life of TNF-α -receptors is longer (Hirota et al 2000).

Also anti-inflammatory cytokines, such as IL-6 (Viedma et al 1992), IL-1Ra and IL-10 (Mayer et al 2000) have predictive value in assessing the severity of AP in the early phase.

A soluble urokinase-type plasminogen activator receptor have recently been detected to serve as a predictive marker of severity in alcohol –induced AP (Nikkola A et al 2017)

Chemokines, such as IL-8 and MCP-1 have predictive value in the development of SAP (Rau et al 1997, Rau et al 2003)

PCT, a marker of sepsis, was detected to have a significant predictive value in the severity assessment in patients with AP (Kylänpää-Back et al 2001). Also, it may predict the development of infected necrosis of pancreas (Mofidi et al 2009). As the infection of the necrotic pancreas is usually seen in the later phase of SAP, the value of PCT as an early predictive marker of infected necrosis is controversial.

Reduced HLA-DR density in monocytes is a sign of decreased immune response (Wolk et al 2000). In patients with AP, low monocyte HLA-DR –expression was found to increase the risk of developing OD (Mentula et al 2003) and secondary infection (Lin et al 2013). For both complications, HLA-DR acts as an early predictive marker.

In terms of systemic manifestations of AP, few “basic” laboratory tests have been found to offer at least moderate prognostic value in predicting the severity of AP. These include serum creatinine (Muddana et al 2009), serum glucose (Mentula et al 2005) and serum calcium, especially when combined with IL-10 (Mentula et al 2005). The value of hematocrit in predicting the severity of AP is controversial (Remes-Troche et al 2006).
2.5.3 Radiology

Computed tomography, especially when intravenous contrast is used, has shown a good accuracy in determining AP (Kivisaari et al 1983). The severity index score, based on findings on CECT, have a good predictive value in mortality and morbidity (Balthazar et al 1990). However, due to progression of necrosis CECT should be done earliest after 72 hours of onset of symptoms (Working Group IAP/APA 2013).

Magnetic resonance imaging can be used as a diagnostic and prognostic tool in AP with similar results to CECT with fewer contraindications (Arvanitakis et al 2007).

Ultrasonography can be used for detection of biliary stones when biliary pancreatitis is suspected. As a diagnostic tool, ultrasonography shows poor results.

2.6 Treatment

There is no specific treatment for AP. Several pharmacological therapies have been studied, but none of them showed clinical benefit (Gardner et al 2008). The treatment is supportive and consists mainly of pain therapy and surveillance for OD. Patients with predicted OD should be admitted to ICU without delay.

Due to hypovolemia produced by fluid loss into third space, early fluid resuscitation has been considered as a standard care for patients with AP. According to the current guidelines, the recommended fluid is Ringer’s lactate with a total infusion rate of 2500-4000 ml within the first 24 hours (Working Group IAP/APA 2013). Indeed, early sufficient fluid resuscitation may prevent the development of SIRS and OD and further, reduce mortality (Gardner et al 2008).

Early enteral nutrition, either oral intake or tube-feeding, is safe and reduces the risk of secondary infections, OD, need for operative interventions and shortens the hospital stay when compared to total parenteral nutrition (Working Group IAP/APA 2013). Therefore early enteral nutrition should be a standard care for patients with AP. Parenteral nutrition should be used only if enteral nutrition fails for any reason.

The current data do not support the use of probiotic therapy in reducing the risk of infectious complications in patients with AP (Gou et al 2014). The current guideline does
not support the use of prophylactic antibiotics in terms of preventing infectious complications (Working Group IAP/APA 2013). However, the issue remains controversial (Lim et al 2015, Ukai et al 2015).

The patient with increased intra-abdominal pressure should be noted and decreased to prevent the development of acute compartment syndrome. According to the current guideline, the preferred treatment is non-surgical, i.e. the use of nasogastric drainage, bowel decompression, restriction of fluid infusion to on-demand –rate, improvement of diuresis and reduction of the abdominal muscle tone. However, if the conservative treatment fails with a sustained intra-abdominal pressure more than 25 mmHg and with a new onset of OD, a surgical intervention is needed (Working Group IAP/APA 2013).

The need for operative treatment in patients with AP is minimal. Patients with biliary pancreatitis complicated with biliary obstruction and/or cholangitis benefit of early endoscopic release of obstruction by ERCP (Working Group IAP/APA 2013). Intervention for necrotizing pancreatitis should be considered, if there is a strong clinical suspicion or confirmation of infection in the necrotic pancreas (Working Group IAP/APA 2013). These include percutaneous drainage and/or mini-invasive endoscopic necrosectomy (step-up approach) or necrosectomy (van Santvoort et al 2010, Working Group IAP/APA 2013). Uncontrolled bleeding, threat of ischemic bowel perforation and gastric–, intestinal–, or biliary obstruction due to the necrotic mass are indications for intervention. In terms of biliary AP, cholecystectomy is recommended and could be done on index admission (mild AP) or delayed 2-6 weeks (more severe AP) (Working Group IAP/APA 2013). Patients unfit for cholecystectomy may benefit for endoscopic sphincterotomy (Working Group IAP/APA 2013).

2.7. Outcome of acute pancreatitis

Patients who have recovered from alcohol-induced AP have a high risk of recurrence. Indeed, 50% develop a new attack, especially if the first one was a mild form (Pelli et al 2000). For the patients with biliary-induced AP, the recurrence risk without cholecystectomy is 18% (Working Group IAP/APA 2013). Pancreatic cells tend to recover after mild AP (Symersky et al 2006). In SAP, the functional recovery depends on
the extent of necrosis. Usually exocrine function recovers after the first AP but permanent impaired glucose metabolism may develop especially in patients with SAP and recurrent AP (Pelli et al 2009, Nikkola J et al 2017)). Morphologic changes consistent with chronic pancreatitis seen in secretin-stimulated magnetic resonance pancreatography remain for half of the patients but these changes are not correlated with pancreatic function (Pelli et al 2009). The majority of the patients who recover from SAP are able to live almost a normal life but their physical strength was still impaired after one year of follow-up (Wright et al 2009). The risk of death after discharge is increased. Of the patients discharged due to SAP, 5% have died within one year at the median age of 65. This may originate from malnutrition, increased risk of infection and exacerbation of underlying comorbid disease. Also the overall survival in patients with SAP is significantly lower when compared to age- and sex –matched population controls (Umapathy et al 2016).

2.8 Leucocyte signaling profiles and trypsinogen-3 in AP

Although leucocyte signaling pathways have been studied extensively in experimental AP, there are only a few studies on clinical AP. Using electrophoretic mobility shift assay, NfKb has been shown to be activated in peripheral blood mononuclear cells (Satoh et al 2003, O’Reilly et al 2006, Raconczay et al 2008). However, the assay is time consuming and requires large amounts of cells and therefore not well-suited for clinical studies. We have applied flow cytometry to study leucocyte signaling in whole blood samples for immunomonitoring in clinical work (Vakkila J et al 2008). This prompted us to study signaling profiles in AP typified by multiple immune aberrations.

Unlike trypsinogen 2, there are no methods to study serum levels of trypsinogen 3 as diagnostic and severity marker of AP.
3 PRESENT INVESTIGATIONS

3.1 AIMS OF THE STUDY

The main goal of the study was to search for novel markers associated with severe acute pancreatitis.

The specific questions to be addressed were as follows:

I) Do peripheral blood monocytes of patients with severe AP show aberrations of intracellular signaling profile

II) Do peripheral blood lymphocytes of patients with severe AP show aberrations of intracellular signaling profile

III) Do peripheral blood polymorphonuclear leucocytes of patients with severe AP show aberrations of intracellular signaling profile

IV) Do serum trypsinogen-3 concentrations associate with severe AP

3.2 MATERIALS

3.2.1 Patients and controls

The studies of leucocyte function (I-III) had 13 (I, III) or 16 (II) patients with SAP. At the time of blood sampling, patients were treated in the ICU at Helsinki University Central Hospital for OD between September 2007 and May 2010. Of the patients (n=16), one had hemodialysis, six had mechanical ventilation and nine had both. All patients were men. All patients could be graded as severe AP due to current classification. The characteristics and outcome of the patients are shown in Table 9.
### Table 9 Characteristics and outcome of the patients (Studies I-III)

<table>
<thead>
<tr>
<th>Character</th>
<th>Median (range)</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td>44 (30-60)</td>
<td>46 (30-74)</td>
</tr>
<tr>
<td><strong>ICU stay, days</strong></td>
<td>20 (7-45)</td>
<td>20 (4-54)</td>
</tr>
<tr>
<td><strong>Respirator Days</strong></td>
<td>20(0-42)</td>
<td>16(1-44)</td>
</tr>
<tr>
<td><strong>Patients, n</strong></td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td><strong>Dialysis Days</strong></td>
<td>10(0-40)</td>
<td>8(0-40)</td>
</tr>
<tr>
<td><strong>Patients, n</strong></td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>RANSON 2,5</td>
<td>2,5 (0-4)</td>
<td>3 (0-5)</td>
</tr>
<tr>
<td>CRP1, mg/ml</td>
<td>193 (80-451)</td>
<td>198 (80-451)</td>
</tr>
<tr>
<td>APACHE II1</td>
<td>15 (6-23)</td>
<td>15 (6-24)</td>
</tr>
<tr>
<td>SOFA2</td>
<td>8 (2-15)</td>
<td>8 (2-17)</td>
</tr>
<tr>
<td>Operation2</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Local complication3</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Infection4</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Mortality</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Abbreviations:
- ICU, intensive care unit; CRP, C-reactive protein; APACHE II, Acute Physiology and Chronic Health Evaluation II; SOFA, Sequential Organ Failure Assessment; ERCP, Endoscopic Retrograde Cholangiopancreatography
- 1 At the day of blood sampling for leucocyte functional studies.
- 2 Including laparotomy and open abdomen/necrosectomy and ECRP
- 3 Including pancreatic necrosis and pseudocyst
- 4 Including sepsis, abdominal abscess and/or pneumonia

The study of trypsinogen-3 comprised of 82 patients (Study IV). They were admitted to the hospital with the median delay from beginning of symptoms of 24 h (range 2 to 72 h) between November 2002 and October 2004. Patients with a delay of more than 72 h were excluded. The blood samples were collected on admission. The characteristics and outcomes of the patients are shown in Table 10.

The diagnosis of AP in all patients was based on typical clinical findings (acute onset of epigastric pain, nausea and vomiting) and elevated serum amylase levels (more than three times the upper reference limit). CECT was performed on all patients in leucocyte function studies within 0-2 days after admission to ICU and to 34 patients in the trypsinogen-3 study upon admission. In addition, to ensure homogenous disease
severity in terms of immunosuppression, an inclusion criterion was that the proportion of HLA-DR-positive monocytes was less than 80%.

The severity of AP was determined according to the previous classification (Table 4). In terms of current classification (Banks et al 2013), the patients with SAP remains in the same severity group due to persistent OD in Study IV. However, the patients classified as mild AP are impossible to re-classify due to current classification because sufficient clinical data is not available (Table 4).

**Table 10. Characteristics and outcome of the patients (Study IV)**

<table>
<thead>
<tr>
<th>Character</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, m/f</td>
<td>59/23</td>
</tr>
<tr>
<td>Age, years, median (range)</td>
<td>50 (19-87)</td>
</tr>
<tr>
<td>Delay of symptoms, hours, median (range)</td>
<td>24 (2-72)</td>
</tr>
<tr>
<td>Etiology</td>
<td></td>
</tr>
<tr>
<td>Alcohol, n</td>
<td>47</td>
</tr>
<tr>
<td>Biliary, n</td>
<td>19</td>
</tr>
<tr>
<td>Other, n</td>
<td>16</td>
</tr>
<tr>
<td>Severity&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Mild, n</td>
<td>57</td>
</tr>
<tr>
<td>Severe, n</td>
<td>25</td>
</tr>
<tr>
<td>Organ dysfunction&lt;sup&gt;1&lt;/sup&gt;, n</td>
<td>6</td>
</tr>
<tr>
<td>APACHE II&lt;sup&gt;2&lt;/sup&gt;, median (range)</td>
<td>6 (0-16)</td>
</tr>
<tr>
<td>SOFA&lt;sup&gt;2&lt;/sup&gt;, median (range)</td>
<td>2 (0-8)</td>
</tr>
</tbody>
</table>

Abbreviations:  
APACHE II, Acute Physiology and Chronic Health Evaluation II;  
SOFA, Sequential Organ Failure Assessment  
<sup>1</sup> According to Atlanta 1992 classification (Bradley 1993)  
<sup>2</sup> At admission to hospital

Sixteen healthy volunteers (median age 45 years, range 25-66, 13 women) served as controls for studies I-III. A total of 63 patients with upper abdominal pain of an etiology other than acute pancreatitis were used as a hospital control group (Table 11). A total of 172 controls participating in the Nordic Reference Interval Project (NORIP) (Felding et al 2000) were used as healthy controls.
Table 11. Final diagnoses of hospital controls (patients with upper abdominal pain and no pancreatitis) (Study IV)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoimmune pancreatitis</td>
<td>1</td>
</tr>
<tr>
<td>Caroli disease</td>
<td>1</td>
</tr>
<tr>
<td>Cholangiocarcinoma</td>
<td>2</td>
</tr>
<tr>
<td>Chronic pancreatitis</td>
<td>3</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>15</td>
</tr>
<tr>
<td>Diverticulitis</td>
<td>1</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>3</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>1</td>
</tr>
<tr>
<td>Hepatocellular cancer</td>
<td>2</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td>3</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>5</td>
</tr>
<tr>
<td>Liver hemangioma</td>
<td>2</td>
</tr>
<tr>
<td>Liver metastasis of other cancer</td>
<td>6</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>8</td>
</tr>
<tr>
<td>Renal cancer</td>
<td>2</td>
</tr>
<tr>
<td>Sepsis</td>
<td>1</td>
</tr>
<tr>
<td>Soft tissue sarcoma</td>
<td>1</td>
</tr>
<tr>
<td>Undefined upper abdominal pain</td>
<td>6</td>
</tr>
</tbody>
</table>

3.2.2 Methods

3.2.2.1 Blood samples

Blood samples (Studies I-III) were collected from the patients within 3 to 5 days after the initial determination of the patients’ monocyte HLA-DR expression status. Each patient had a reference subject of his own. Parallel peripheral blood samples were obtained from the patient and his reference control on two consecutive days. On day 1, a 4-ml sample of peripheral venous blood was taken (i) for the study of phosphorylation
promoted by soluble leucocyte agonists and (ii) for the re-study of the proportion of HLA-DR-positive monocytes. In addition, 40 ml sample of peripheral venous blood was taken for monocyte and PMNL transmigration assay on day 1. On day 2, a 4-ml blood sample was taken for the study of leucocyte phosphorylation in response to bacterial cells. The blood samples were collected into Falcon polypropylene tubes (Becton Dickinson, Lincoln Park, NJ) containing pyrogen-free citrate phosphate dextrose (ACD, Baxter Health Care Ltd, Norfolk, England, 0.14 ml/ml blood), kept at the room temperature, and transported within 15 min to the laboratory. All aliquots of the blood samples were stimulated within 4 h of sampling.

While the lymphocyte study was in progress the patients’ lymphocytes showed constitutive STAT3 activation, that is, STAT3 RFU-values in resting cells were consistently higher in patients than controls. The original protocol was adapted to measure constitutive STAT3 activation in all lymphocytes and lymphocyte subsets. The revised protocol was applied to patients 11-13 and the STAT3 part of the revised protocol to patients 14-16.

Blood samples for preparation of serum (Study IV) for determination of trypsinogen-3 were collected from the patients on admission to hospital. For control group, serum samples were obtained from 172 apparently healthy controls participating in the Nordic Reference Interval Project (NORIP) and from 63 patients with upper abdominal pain of an etiology other than acute pancreatitis (hospital controls). The AP and control samples were stored at -20°C before the determination of trypsinogen-3 concentrations for 2.6-4.4 years (median 3.6 years) and 3.5-5.0 years (median 4.4 years), respectively. The stability of trypsinogen-3 was studied using 11 serum samples from AP patients. After the assay, these samples were stored for 3.7 years at -80°C before re-assay.

3.2.2.2 Flow cytometry (I-III)

Fleisher et al (Fleisher et al 1999) was the first to use phosphor-specific flow cytometry. To minimize ex vivo activation of signalling pathways brought about by blood sample handling, such as leukocyte separation procedures, our group applied flow cytometry to study monocyte STAT1 phosphorylation in whole blood samples (Vakkila et al 2008). In
the present thesis, the method was extended to studies of neutrophils and lymphocytes and intracellular signalling pathways NF-kB, p38 and STAT3.

Antibodies and leucocyte-stimulating agents

Monoclonal antibodies used in Studies I-III are showed in Table 12.

Table 12. Monoclonal antibodies

<table>
<thead>
<tr>
<th>Against cell surface structures</th>
<th>Study I</th>
<th>Study II</th>
<th>Study III</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC/CD14 clone MfP9 (IgG2b)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FITC/CD19 clone Sj25C1 (IgG1)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>FITC/CD4 clone SK3 (IgG1)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PerCP/CD45 clone 2D1 (IgG1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PerCP/CD3 clone SK7 (IgG1)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PE/anti-HLA-DR clone L243 (IgG2a)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Isotype control (mouse IgG2a)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phospho-specific</th>
<th>Study I</th>
<th>Study II</th>
<th>Study III</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNFkB p65 (pS529) clone K10-895.12.50 (IgG2b)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>STAT1 (pY701) clone 4a (IgG2a)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ERK1/2 (pT202/pY204) clone 20A (IgG1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PE/pp38 (pT180/Y182) clone 36 (IgG1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>STAT3 (pY705) clone 4/p-STAT3 (IgG2a)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>STAT5 (Y694) clone 47 (IgG1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>STAT6 (Y641) clone 18 (IgG2a)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Abbreviations:
CD, cluster of differentiation; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; HLA-DR, human leucocyte antigen –DR; IgG, immunoglobulin G; NFkB, nuclear factor kB; PE, phycoerythrin; PerCP, peritidin-chlorophyll-protein complex; STAT, signal transducer and activator of transcription

All antibodies were purchased from Becton-Dickinson Biosciences (San Jose, CA).

The recombinant cytokines TNF (Studies I-III), IL-4 (Study II) and IL-6 (Studies I-III) were purchased from R&D (Minneapolis, MN). fMLP, PMA (Studies I-III), calcium ionophore A23187 (Studies I-III), E. coli O11:B4 LPS (Studies I-III), MDP (Studies I-III), and MCP-1 (Study I), were purchased from Sigma (St.Louis, MO). GM-CSF (Study III) was purchased from Schering-Plough Corporation (Kenilworth, NJ).
*S. aureus* (IHT 61972) (Studies I-III) and *E. coli* (IH 3080) (Studies I-III) were obtained from the National Institute for Health and Welfare, Helsinki, Finland. *S. epidermidis* (Studies I-III) was the strain ATCC 53103. Each strain was grown in a brain heart infusion medium. The bacteria were pelleted and washed twice. A diluted culture was made to quantify viable bacteria. The bacteria were pelleted and re-suspended in a glycerol-tryptone soya broth medium and stored at -70 °C.

*Ex vivo stimulation and immunolabeling of blood samples for three-colour flow cytometry*

The 4-ml blood sample was divided into 90 ml aliquots in polystyrene tubes (Becton Dickinson, Lincoln Park, NJ) and placed at 37 °C. Aliquots of FITC-conjugated mAbs to CD14 (Studies I-III), CD4 (Study II) and CD8 (Study II) were added into the tubes. Next the tubes were supplemented either with TNF 10 ng/ml and incubated for 5 min at 37 °C (Studies I-III), LPS 100 ng/ml for 10 min (Studies I-III), MDP 100 ng/ml for 20 min (Studies I-III), IL-6 100 ng/ml for 5 min (Studies I-III), IL-4 100 ng/ml 5 min (Study II), GM-CSF 5 ng/ml for 5 min (Studies I and III), combination of PMA 1 mM and calcium ionophore A23187 mM for 5 min (Studies I-III), *E. coli* 50 cells/leucocyte for 10 min (Studies I-III), *S. aureus* 50 cells/leucocyte for 20 min (Studies I-III) or *S. epidermidis* 50 cells/leucocyte for 40 min (Studies I-III). In Study II, one series of infliximab (10mg/ml) or anakinra (100mg/ml), or both were mixed with the blood obtained from healthy volunteers before addition of bacteria. The number of stimulated samples was 13 in Studies I and III. In Study II the number of stimulated samples was as follows: TNF (10 patients), IL-6 (13 patients), IL-4 (10 patients), PMA+ calcium ionophore A23187 (10 patients), LPS (10 patients), MDP (10 patients), *E. coli* (10 patients), *S. aureus* (nine patients), *S. epidermidis* (nine patients), respectively. Reference tubes were left without stimulus. The dose-response and time-course of phosphorylation were determined for each stimulus using blood samples from healthy volunteers.

After incubation, a 1x BD PhosFlow Lyse/Fix Buffer (1.9 ml) pre-warmed to 37°C was added to each tube, they were then incubated for 10 min at 37°C, and washed once with Hank’s balanced salt solution (Life Technologies, Paisley, UK). For permeabilization, the cell pellet was re-suspended in 1 ml of BD Phosflow Perm Buffer III, pre-cooled at -20°C. The tubes were stored at -20°C until staining with phosphospecific mAbs. After permeabilization, the cell samples were washed twice with ice-cold BD Pharmingen Stain Buffer and re-suspended in 100 μl of the buffer. Aliquots of Alexa647- and PE-
labelled phosphospecific mAbs were then added to the stimulus-treated samples and respective reference samples. In addition, aliquots of CD3-PerCP mAb were added at this stage in Study II, because PerCP did not withstand the permeabilization procedure. All the samples were further incubated in the dark for 30 min at 0°C, washed once, and re-suspended in 500 μl of the ice-cold stain buffer. The samples were stored at 0°C and analyzed by flow cytometry within 3 hours.

Data acquisition and analysis were done with a FACS Calibur flow cytometer and Cell Quest software (BD Sciences, San Jose, CA). Lymphocytes and PMNLs were delineated according to their light scattering properties using electronic gates. Monocytes were recognized by CD14-FITC label positivity. CD19-FITC-label was used to identify B-lymphocytes and the CD3-PerCP-label was used in combination with the CD4-FITC- or CD8-FITC-labels to identify CD3+CD4+T lymphocytes and CD3+CD8+T lymphocytes.

Data are presented as median fluorescence intensity, that is median or mean relative fluorescence units (RFU) of the whole studied cell population, as a proportion (%) of positively fluorescing cells, and as a mean fluorescence intensity of the positively fluorescing cells (Studies I-II). The percentage of fluorescing cells was measured using a threshold method, where an electronic gate was set manually so that it included the brightest 2 to 4% of the cells in non-stimulated sample. Thus, the values < 5% indicate that the cells have not responded to the stimulus. The coefficient of variation was 5% within experiments and 10% between experiments. Monocyte surface expression of HLA-DR, in other words the proportion (%) of monocytes positive for HLA-DR fluorescence, was determined.

A total of 2x10^3 CD14-positive monocytes (Study I), 1x10^4 lymphocytes, 2x10^3 CD3+CD4+ T lymphocytes, 2x10^3 CD3+CD8+ T lymphocytes, and 2x10^3 CD19+B lymphocytes (Study II) and 1x10^4 PMNLs were collected and appropriate Alexa647- and PE-histograms were developed from them. In addition, during study II, we saw that in a proportion of the patients’ lymphocyte STAT3 was constitutively active. To determine the proportion of lymphocytes showing constitutive activated STAT3 lymphocytes, the electronic gate of non-stimulated lymphocytes of the controls studied concomitantly was used in the analysis of the patients’ samples. In study III, to determine the proportion of PMNLs showing constitutively activated STAT3, the electronic gate of non-
stimulated PMNLs of the healthy subject studied concomitantly was used in the analysis of the patients’ samples.

3.2.2.3 Transendothelial migration assay (I, III)

A confluent endothelium was cultured from EA-HY cell line on Transwell cell culture inserts (5 mm pores, Biocoat, Corning, NY) in D-MEM with 10 % fetal bovine serum (Gibco, Auckland, NZ), 50mg/ml penicillin, 50U/ml streptomycin (both from Sigma, St.Louis, MO) and 5% CO2 at 37 °C. The 40 ml blood samples from a healthy subject and a patient were incubated at 37 °C for 1h, the buffy coat fraction was collected, and mononuclear leucocytes were separated using Ficoll-Paque (GE Bio-Sciences, Uppsala, Sweden; centrifugated 1500 rpm for 30 minutes). The mononuclear fraction was collected and mononuclear cells were calculated in Brücker’s chamber.

Aliquots of mononuclear cells (400000/well in Study I; 250000/well in Study III) in Dulbecco’s phosphate buffered saline (DPBS) with calcium (0.9mM) and magnesium (0.5mM, Gibco, Auckland, NZ) were applied on to the confluent endothelium layer in the upper chamber of Transwell. The lower chamber was used with or without MCP-1 (5nmol/l) (Study I) or with or without fMLP (50 nmol/l) (Study III) in DPBS. The experiments were done in triplicates. After three (Study I) or two (Study III) hours incubation the lower chamber liquid was collected and centrifuged. The cells were washed, placed in Trucount™ tubes (BD Bioscience, San Jose, CA) and labeled with CD14-FITC (Study I) or CD15-FITC (Study III) and CD45-PE antibodies. Monocytes were recognized by CD14/CD45 staining and monocyte light scattering properties. PMNLs were recognized by CD15/CD45 staining and light scattering properties. The advantage of Trucount™ pearls is that they provide a means to determine the absolute count of cells in a known volume. The applied and transmigrated amount of monocytes or PMNLs (cells/ml) was calculated.

The results are given as proportion of transmigrated cells (%), i.e., transmigration % = 100 x count of cells transmigrated through the endothelial epithelium and Transwell membrane / count of cells applied to the upper compartment of the Transwell. The Transwell pore size, incubation period in transmigration and amount of monocytes or PMNLs were adjusted in preliminary experiments to provide maximum response.
3.2.2.4 Immunofluorometric assay for trypsinogen-3 (IV)

Trypsinogen-3 was determined by a sandwich-type immunoassay (Itkonen et al 1990) using Mab F136-13E6 for capture and MAb F141-5F3 labeled with a Eu-chelate (Perkin-Elmer, Turku, Finland) as a tracer. Twenty five µL of calibrators or serum samples together with 200 µL assay buffer were incubated in microtiter plate wells coated with capture antibody (10 mg/L) for 1 h, followed by washing and addition of Eu-labeled tracer antibody (100 ng/well in 200 µL assay buffer) for 0.5 h. Calibrators containing 1.0-250 µg/L of trypsinogen-3 were prepared in assay buffer. As calibrators we used trypsinogen-3 with Lys23Gln mutation to prevent activation of the proenzyme. The content of trypsinogen-3 was determined on the basis of the absorbance at 280 nm using an extinction coefficient of 1.6, determined by the ProtParam tool of ExPASy Proteomics Server. Intra- and inter-assay coefficients of variation of the assay were determined from 9 and 12 replicates, respectively, using serum samples containing 1.5-110.4 µg/L of trypsinogen-3.

3.2.3 Statistical analysis

In Studies I-II, results are shown in mean±SEM or median (range). Statistical comparisons between the groups were carried out using the nonparametric Wilcoxon-Mann-Whitney test.

Because PMNLs (Study III) appeared not to respond to each of the stimuli used in the test system, we initially evaluated significance of difference in fluorescence intensities between stimulated and non-stimulated samples of controls (n=13, Wilcoxon-Mann-Whitney test, p<0.05). Signaling proteins whose fluorescence intensities differed significantly were selected for analysis of the patients’ samples. The data are shown as mean±SEM or median (range). Statistical comparisons between the groups were carried out using nonparametric Wilcoxon-Mann-Whitney test and Wilcoxon signed rank test.

In Study IV, reference values were calculated separately for females and males and for age groups 18-30, 31-50 years, 51-70 years and >70 years using Analyse-it for Microsoft® Excel 2003 (version 2.04, Analyse-it Software, Ltd., Leeds, UK). Statistical
comparisons and correlation analysis between the groups were carried out using Mann-Whitney-U test and Spearman’s correlation analysis (SPSS Statistics 17.0, IBM Corporation, NY). Receiver operating characteristics (ROC) curve and linear regression analyses were carried out by using Analyse-it. Results below the limit of quantitation (1.0 µg/L) were assigned a value of 0.5 µg/L.

Probabilities were regarded as statistically significant at the level of 0.05 in all studies.

3.2.4 Ethical aspects

The study protocols were approved by the Surgical Ethical Review Board of the Joint Authority for the Hospital District of Helsinki and Uusimaa, and informed consent was obtained from each patient, or from next of kin of the patient.

3.3 RESULTS

3.3.1 Signaling profiles of blood leucocytes

3.3.1.1 Monocytes (I)

NFkB phosphorylation

The levels of pNFkB p65 of the patient monocytes stimulated by six different agonists were significantly lower than in controls ($p<0.002$) (Fig 4). The proportion of phosphorylated monocytes and their mean fluorescence intensities (RFU) were also significantly lower in patients than in controls (Table 12). This implies that both the proportion of responding monocytes and their reaction capacity were decreased in the patients.

Each stimulus used induced a strong p38 phosphorylation in both patients and controls. Neither stimulated nor nonstimulated pp38 levels differed significantly between patients and controls. In addition, the proportions of pp38-positive monocytes and their fluorescence intensity values in patients and controls were comparable, with the exception of the samples treated with S. aureus, where the proportion of pp38-positive monocytes was higher in the patients ($84.3 \pm 2.9\%$ vs. $66.8 \pm 4.2\%, p = 0.011$).
Levels of NF-kB p65 phosphorylation in all the CD14-positive monocytes of controls (circles) and patients (quadrangles) with acute pancreatitis in whole blood samples without supplement (open symbols) or supplemented (closed symbols) with TNF (10 ng/ml, 5 min), LPS (100 ng/ml, 10 min), MDP (100 ng/ml, 20 min) in the upper panel and *E. coli*, *S. aureus* or *S. epidermidis* in the lower panel. The stimulated p65 phosphorylation levels were lower in patients than controls (for each stimulus p<0.002, N=8-10). Horizontal lines denote medians.
Table 13. Proportion (%) and mean RFU of pNFκB positive cells during stimulation

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Patients</th>
<th>Controls</th>
<th>p-value</th>
<th>Patients</th>
<th>Controls</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>40.1±4.7</td>
<td>80.8±3.3</td>
<td>p&lt;0.001</td>
<td>19±1</td>
<td>34±2</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>LPS</td>
<td>30.5±4.0</td>
<td>72.9±3.6</td>
<td>p&lt;0.001</td>
<td>17±1</td>
<td>29±2</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>MDP</td>
<td>17.7±7.5</td>
<td>29.1±3.8</td>
<td>p=0.043</td>
<td>16±1</td>
<td>22±1</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>E. coli</td>
<td>48.4±5.9</td>
<td>91.7±1.6</td>
<td>p&lt;0.001</td>
<td>20±2</td>
<td>36±3</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>S. aureus</td>
<td>45.0±5.0</td>
<td>59.8±3.2</td>
<td>p=0.024</td>
<td>18±2</td>
<td>28±2</td>
<td>p=0.002</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>38.3±6.4</td>
<td>79.0±3.3</td>
<td>p&lt;0.001</td>
<td>16±2</td>
<td>26±2</td>
<td>p=0.006</td>
</tr>
</tbody>
</table>

Abbreviations:
LPS, lipopolysaccharide; TNF, tumor necrosis factor;
The levels of NF-kB p65 phosphorylation (RFU) in all the CD14-positive monocytes are presented in Fig.4.

**STAT phosphorylation**

IL-6 stimulated blood samples from the patients showed lower STAT1 (Figure 5A) and STAT3 (Fig 5B) phosphorylation levels than did those from controls (both p < 0.001). Indeed, the level of STAT1 phosphorylation in IL-6 –stimulated or non-stimulated cells from the patients were comparable (5.0 ± 0.2 RFU vs. 6.0 ± 0.4 RFU, p > 0.05). The proportions of STAT1-positive monocytes and their relative fluorescence intensities were lower in patients than in controls (6.7 ± 2.7% vs. 51.8 ± 5.3%, p < 0.001 and 14 ± 1 RFU vs. 17 ± 1 RFU, p = 0.017). The proportions of STAT3-positive monocytes and their relative fluorescence intensities were also lower in patients than in controls (75.3 ± 5.7% vs. 97.3 ± 0.3% and 33 ± 4 RFU vs. 58 ± 7 RFU, both p < 0.001). There were no statistically significance differences in pSTAT5 levels between the patients and controls.
Levels of STAT1 (A) and STAT3 (B) phosphorylation in all the CD14-positive monocytes of controls (circles) and patients (quadrangles) in whole blood samples without supplement (open symbols) or supplemented (closed symbols) with IL-6 (100 ng/ml, 5 min). The IL-6-stimulated STAT1 and STAT3 phosphorylation levels were lower in patients than controls (both p-values<0.001, N=9-13). Thin horizontal lines denote medians.

**ERK1/2 phosphorylation**

The patients’ monocytes treated with PMA and calcium ionophore showed lower pERK1/2 levels than did similarly treated monocytes of controls (p < 0.05) (Fig 6). The proportion of pERK1/2 positive monocytes was lower in patients than controls but the difference was not statistically significant (48.3 ± 7.2% vs. 53.0 ± 7.5%, p > 0.05). However, the mean RFU of the pERK1/2 positive monocytes was significantly lower in patients than in healthy controls (30 ± 2 RFU vs. 37 ± 2 RFU, p = 0.016).
Levels of ERK1/2 phosphorylation in all the CD14-positive monocytes of healthy subjects (N=9, circles) and patients (N=10, quadrangles) were measured in whole blood samples without supplement (open symbols) or supplemented (closed symbols) with combination of PMA (1 mM) and Ca-ionophore (1 mM). The stimulated phosphorylation levels were lower in patients than controls (p=0.011). Thin horizontal lines denote medians.

3.3.1.2 Lymphocytes (II)

NFκB phosphorylation

Stimulation with TNF induced increased phosphorylation of NFκB among all lymphocytes, CD3⁺CD4⁺ and CD3⁺CD8⁺-lymphocytes in controls. In TNF-stimulated samples, NFκB phosphorylation levels were lower in patients than in controls among all lymphocytes, CD3⁺CD4⁺ and CD3⁺CD8⁺-lymphocytes. (Fig 7A). In addition, the proportions of pNFκB-positive lymphocytes and the fluorescence intensities of them were significantly lower in patients than in controls among all lymphocytes, CD3⁺CD4⁺– and CD3⁺CD8⁺–lymphocytes (Fig 7B). Stimulation with whole bacteria, but not bacterial structures (LPS, MDP), promoted NFκB phosphorylation among all lymphocytes of controls. The fluorescence intensity values of NFκB among all lymphocytes were significantly lower in patients than in controls (Fig 7C). When the blood samples were treated with E. coli or S. epidermidis, the proportions of pNFκB-positive lymphocytes among the whole lymphocyte population were significantly lower in patients than in
controls. (Fig 7D). The fluorescence intensities of pNFκB –positive lymphocytes of the patients and controls were comparable.

The fluorescence intensities of pp38 in all lymphocytes were significantly higher in patients than in controls, when the cells were stimulated by TNF (3.4 ± 0.5 RFU vs. 2.5 ± 0.1 RFU, p<0.05), E. coli (2.7 ± 0.2 RFU vs. 2.0 ± 0.1 RFU, p<0.01) or S. aureus (2.9 ± 0.3 RFU vs. 2.1 ± 0.1 RFU, p<0.05). The proportions of pp38 –positive lymphocytes were higher in patients’ samples than in controls’ samples, when the samples were stimulated by E. coli (15.6 ± 1.8% vs. 2.0 ± 0.1%, p<0.01) or S. aureus (16.0 ± 3.5% vs. 6.1 ± 0.6%, p<0.05). The fluorescence intensities of pp38 –positive cells among all lymphocytes were higher in patients than in controls, when the blood samples had been treated with TNF, whole bacteria or bacterial structures (LPS or MDP), but the differences were not statistically significant (data not shown).
Figure 7.

Levels of phosphorylated NF-kB p65 (pNF-kB) in lymphocytes (Ly) of controls (circles) and patients (quadrangles) with acute pancreatitis were measured in whole blood samples left without supplement (open symbols) or supplemented (closed symbols) with TNF (10 ng/ml, 5 min), E. coli, S. aureus, S. epidermidis, LPS, or MDP. Responses to TNF, determined as: A. fluorescence intensity (FI) of all Ly and subsets of Ly (N=9-10). B. as a proportion of pNFkB-positive Ly and their FI. C. Responses to bacteria, LPS, and MDP, determined as the FI of all Ly (N=8-10). D. as proportion of pNFkB-positive cells among all Ly and their FI.

RFU, relative fluorescence units. In A and C, horizontal lines denote the median. In B and D, data are given as mean±SEM. *p < 0.05, **p < 0.01, *** p < 0.001.
ERK1/2 phosphorylation

When the blood samples were treated with a combination of PMA and calcium-ionophore, pERK1/2 fluorescence intensities were lower in patients’ than in controls among all lymphocytes ($p < 0.05$) (Fig 8). The proportion of pERK1/2 positive lymphocytes was lower in patients ($22.8 \pm 7.8\%$ vs. $43.3 \pm 5.1\%, p = 0.043$). There was no significant difference in fluorescence intensities of pERK1/2 –positive lymphocytes between patients and controls ($12.2 \pm 0.6$ RFU vs. $11.5 \pm 0.6$ RFU, $p > 0.05$).

Figure 8.

Levels of ERK1/2 phosphorylation in lymphocytes of controls (circles) and patients (quadangles) were measured in whole blood samples without supplement (open symbols) or supplemented (closed symbols) with combination of PMA (1 mM) and Ca-ionophore (1 mM). RFU, relative fluorescence units. *$p < 0.05$ ($N = 8-10$).

STAT phosphorylation

The patients’ non-stimulated lymphocytes showed significantly higher fluorescence intensities of pSTAT3 than did the controls’ respective cells ($p < 0.001$, Fig 9A). The shapes of the patients’ pSTAT3 –histograms were biphasic (Fig 9B). The proportion of non-stimulated pSTAT3 positive cells was higher in patients than in controls ($p < 0.001$, Fig 9C). When the cells were stimulated by IL-6, the pSTAT3 fluorescence intensities of lymphocytes of patients and controls were comparable. The lymphocyte subgroup
analysis of non-stimulated cells showed that patients’ CD3+CD4+ lymphocytes, as compared with controls respective cells, had higher pSTAT3 levels, as defined by pSTAT3 fluorescence intensity of all CD3+CD4+ cells (Fig. 9D), the proportion of pSTAT3-positive cells (Fig. 9E) and pSTAT3 fluorescence intensity of pSTAT3-positive cells (Fig. 9F). The proportion of pSTAT3 positive CD+CD8+ lymphocytes was higher in patients than in controls (Fig. 9E).
Levels of phosphorylated STAT3 (pSTAT3) in lymphocytes of controls (circles) and patients (quadrangles) in whole blood samples without supplement (open symbols) or supplemented (closed symbols) with IL-6 (100 ng/ml, 5 min). A. Fluorescence intensity (RFU, relative fluorescence units); B. sample histograms; C. proportion of pSTAT3-positive cells among all lymphocytes (N = 12-13); D. Fluorescence intensity; E. proportion of pSTAT3-positive lymphocytes among; F. fluorescence intensity of pSTAT3-positive lymphocytes among subsets of non-stimulated lymphocytes. In B, M1 (marker) denotes the proportion of pSTAT3-positive cells. Horizontal lines in A and C-F denote the median and M1 (marker) in B denotes the proportion of pSTAT3-positive lymphocytes. *p < 0.05, **p < 0.01, ***p < 0.001.
The patients had lower IL-6 induced pSTAT1 levels than did the controls, as determined by fluorescence intensity of all lymphocytes, although the difference was not significantly significant ($p = 0.058$, Fig 10A). The proportion of pSTAT1- positive cells and fluorescence intensity of pSTAT1 positive cells were lower in patients than in healthy controls ($p < 0.05$, Fig 10C).

When the cells were stimulated by IL-4, pSTAT6- fluorescence intensities were higher in patients’ lymphocytes than in controls’ lymphocytes (Fig 10B). The proportion and the fluorescence intensity of pSTAT6- positive lymphocytes were higher in patients than in controls, but the differences were not significant (Fig 10C).

**Figure 10.**

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>% of pSTAT-positive Ly</th>
<th>pSTAT1</th>
<th>pSTAT6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy subject</td>
<td>IL-6</td>
<td>IL-4</td>
</tr>
<tr>
<td>% of pSTAT-positive Ly</td>
<td>Patient</td>
<td>13.8 ± 1.6</td>
<td>47.6 ± 5.1</td>
</tr>
<tr>
<td>FI (RFU) of pSTAT-positive Ly</td>
<td>Healthy subject</td>
<td>11.4 ± 0.4</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Patient</td>
<td>9.7 ± 0.2</td>
<td>6.3 ± 0.3</td>
</tr>
</tbody>
</table>

Levels of A. pSTAT1 fluorescence intensity (FI); B. pSTAT6 FI in lymphocytes (Ly) of controls (circles) and patients (quadrangles) in whole blood samples left without supplement (open symbols) or supplemented (closed symbols) with IL-6 (100 ng/ml, 5 min) in A and IL-4 (100 ng/ml, 5 min) in B. C. The proportions of pSTAT1- or pSTAT6-positive Ly and their FI. RFU, relative fluorescence units. *$p < 0.05$. 

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3.3.1.3 PMNLs (III)

**NFκB phosphorylation**

The proportions of pNFκB–positive PMNLs were significantly lower in patients than controls when samples were stimulated by TNF ($p = 0.004$) (Fig. 11). The fluorescence intensities of all PMNLs and pNFκB–positive PMNLs of the patients and those of the controls were comparable (data not shown).

**Figure 11.**

Proportion of pNFκB positive polymorphonuclear leucocytes (PMNLs) of healthy subjects (circles) and patients (quadangles) with acute pancreatitis in whole blood samples without supplement (open symbols) or supplemented (closed symbols) with TNF (tumor necrosis factor 10 ng/ml, 5 min), E.coli (Escherichia coli), S.aureus (Staphylococcus aureus), LPS (lipopolysaccharide) and stained with mAbs against CD14 and pNFκB. Horizontal lines denote medians. N=9-10.

The levels of pp38 intensities of PMNLs, without stimulus or stimulated by four different agonists, were comparable in and controls. Also the proportions of pp38–positive PMNLs and the fluorescence intensities of pp38–positive PMNLs, with or without stimulus, were comparable (data not shown)
**ERK1/2 phosphorylation**

The proportions of pERK1/2–positive PMNLs stimulated with the combination of calcium ionophore and PMA were significantly lower in patients than in controls \((p = 0.027)\) (Fig. 12). The fluorescence intensities of PMNLs and pERK1/2–positive PMNLs of the patients and controls were comparable (data not shown).

**Figure 12.**

The proportion of ERK1/2 phosphorylation (pERK1/2)–positive polymorphonuclear leucocytes (PMNLs) of controls (circles) and patients (quadrangles) were measured in whole blood samples without supplement (open symbols) or supplemented (closed symbols) with combination of PMA (phorbol-12-myristate-13-acetate 1 mM) and Ca-ionophore (1 mM). The proportion of pERK1/2 positive cells was slightly decreased in patients as compared to controls \((p=0.027, N=8-10)\). Horizontal lines denote medians.

**STAT phosphorylation**

In nonstimulated blood samples, the proportion of pSTAT3–positive PMNLs of a total of five out of 13 patients was higher than the highest proportion of pSTAT3–positive PMNLs of 12 controls. (Fig 13). In samples treated with IL-6, the proportion of pSTAT3–positive PMNLs was significantly higher in patients than in controls \((38.1 \pm 8.4\% \text{ vs.})\).
10.7 ± 2.2%, $p = 0.016$, Fig 13). Also the fluorescence intensity of pSTAT3 -positive PMNLs tended to be higher in patients than in controls but the difference was not statistically significant (24.7 ± 1.6 RFU vs. 20.7 ± 0.7 RFU, $p = 0.075$)

After stimulation with GM-CSF, the proportions of pSTAT5 -positive PMNLs and the fluorescence intensities of pSTAT5 -positive PMNLs of patients and controls were comparable (data not shown).

After stimulation with IL-4, the pSTAT6 fluorescence intensity values of PMNLs from patients were lower than those from controls (7.7 ± 0.3 RFU vs. 9.1 ± 0.4 RFU, $p = 0.011$, Fig 14). The proportion of pSTAT6 and the fluorescence intensity of pSTAT6 -positive PMNLs from patients and controls were comparable (data not shown).

**Figure 13**

Phosphorylation of STAT3 (pSTAT3) in polymorphonuclear leucocytes (PMNLs) of controls (circles) and patients (quadrangles) in whole blood samples without supplement (open symbols) or supplemented (closed symbols) with IL-6 (interleukin-6, 100 ng/ml, 5 min). The proportion of pSTAT3 positive cells induced by IL-6 was higher in patients than in controls ($p=0.016$, N=12-13. Horizontal lines denote medians.
Phosphorylation of STAT6 (pSTAT6) in polymorphonuclear leucocytes (PMNLs) of controls (circles) and patients (quadrangles) in whole blood samples without supplement (open symbols) or supplemented (closed symbols) with IL-4 (interleukin-4, 100 ng/ml, 5 min). pSTAT6 (RFU, relative fluorescence intensity) of all PMNLs in IL-4 stimulated samples were lower in patients than in controls (p=0.011, N=9-10). Horizontal lines denote medians.

3.3.2 Transendothelial migration of monocytes and PMNLs (I, III)

The proportion of patients’ monocytes that transmigrated through the endothelium was significantly lower than that of controls’ monocytes  (2.9 ± 0.8% vs. 6.2 ± 1.2%, p = 0.036, Fig 15A)

The proportion of the patients PMNLs that transmigrated through the endothelium was significantly higher than that of controls’ PMNLs (47.8 ± 7.2% vs. 34.3 ± 4.9%, p = 0.023, Fig 15B)
Transmigration of monocytes (A) and polymorphonuclear leucocytes (PMNLs) (B) from controls (N=8 in A and B; circles) and patients (N=9 in A; N=8 in B; quadrangles) through cultured endothelial monolayers without attractant (open symbols) or with (A) MCP-1 (5 nmol/l, closed symbols) or (B) FMLP (formyl-methionyl-leucyl-phenylalanine, 50 nmol/l, closed symbols). Horizontal lines denote medians. In A) the difference between MCP-1 treated monocytes of patients and controls was significant (p=0.036); in B) the difference between FMLP treated PMNLs of patients and controls was significant (p=0.023)

3.3.3 Trypsinogen-3 (IV)

The sandwich-type immunoassay using Mab F136-13E6 as capture antibody and F141-5F3 as tracer recognized both trypsinogen-3 and trypsin-3. Cross-reactivity with the other trypsinogens was < 0.1%. The trypsinogen-3 assay was specific for trypsinogen-3 as determined by separation of the different trypsinogen isoenzymes in serum from AP patients by anion exchange chromatography and analysis of the separated fractions by time-resolved immunofluorometric assays for trypsinogen-1, -2 and -3 (Fig 16). The calibration curve was linear over the range of 1.0 – 250 µg/l (Fig 17). The lower limit of detection was 0.4 µg/l, whereas the lower limit of quantification was 1.0 µg/l. The
recovery of trypsinogen-3 concentration in serum samples was 89% (23%) (range of 28-114%) after sample storage for 3.7 years at - 80°C.

Figure 16.

Fractions of trypsinogen isoenzymes from separated sera of patients with AP

Figure 17.

Calibration curve and precision profile (CV, n = 5) with recombinant Lys 23Gin trypsinogen-3 as calibrator.

Reference intervals were established with 172 serum samples from healthy controls. There were no differences in trypsinogen-3 concentrations between genders or according to age. The calculated upper reference limit of the 97.5 percentile was 4.4 µg/l with a range of < 1.0 – 13.5 µg/l.
The median concentration of trypsinogen-3 was significantly higher in patients with mild AP (9.5 µg/l, range 7.3 – 20.5 µg/l, \( p < 0.0001 \)) and in patients with SAP (15 µg/l, range 5.5 – 20.7 µg/l, \( p < 0.0001 \)) than in the hospital control group (< 1.0 µg/l, >1.0 – 1.2 µg/l). The patients with alcohol-induced AP showed significantly higher trypsinogen-3 concentrations (median 17.4 µg/l, range <1.0 – 195 µg/l) compared with biliary-induced AP (median 6.7 µg/l, range < 1.0 – 45.1 µg/l, \( p = 0.015 \)). Also in samples from the patients with recurrent AP the median concentration of trypsinogen-3 was significantly higher in (20.6 µg/l, 8.4 – 23.4 µg/l, \( p < 0.0001 \)) than in the hospital control group. There was no statistically significant difference in trypsinogen-3 concentrations between mild and SAP (\( p > 0.75 \)). ROC analysis showed, that the area under curve (AUC) of trypsinogen-3 for separation between patients with AP and the hospital control group was 0.90 (\( p < 0.0001 \)) (Fig 18), in mild AP 0.88 (\( p < 0.0001 \)), in SAP 0.93 (\( p < 0.0001 \)) and in recurrent AP 0.94 (\( p < 0.0001 \)), respectively. There was no relationship between serum trypsinogen-3 and amylase in AP (\( R^2 = 0.02, p = 0.169 \)), mild AP (\( R^2 = 0.01, p = 0.372 \)) or SAP (\( R^2 = 0.09, p = 0.136 \)).

**Figure 18.**

ROC curve for trypsinogen-3 for separation between patients with AP and control group. Abbreviation: AUC, area under curve.
3.4 DISCUSSION

3.4.1 Leucocyte signaling and transendothelial migration (I-III)

The results of the Studies I-III show, for the first time, multiple aberrations in pro- and anti-inflammatory signaling pathways of leucocytes, as determined by phospho-specific whole blood flow cytometry, in patients with AP complicated by end organ dysfunction and immune suppression.

NFκB

The stimulation of the patients’ monocytes by *E. coli*, *S. aureus*, and *S. epidermidis* showed strongly depressed NFκB activation. In addition, when patients’ lymphocytes were stimulated with exogenous TNF, NFκB phosphorylation was depressed. According to the histograms with uniform shape, the defects appeared to involve all circulating monocytes and lymphocytes. In PMNLs, the proportions of pNFκB-positive cells were lower in patients than in controls in response to TNF and tended to be lower in response to *E. coli* and *S. aureus*.

These results are in line with previous study (Satoh et al 2003) indicating that NFκB activation of peripheral blood mononuclear cells in response to LPS was depressed, as determined by electrophoretic mobility shift assay. Taken together, these findings are in accordance with the results indicating that patients with end organ dysfunction associated with systemic inflammation have poor NFκB-mediated innate immune responses *in vivo* to verified bacterial infections (Headley et al 1997). The impaired collaboration of both monocytes and lymphocytes with a significant reduction of circulating T- and B-lymphocytes in SAP (Pietruczuk et al 2006) may increase the risk of development of secondary infections. The decreased proportion of pNFκB-positive PMNLs may originate from their retained capacity to emigrate to tissues. However, their phosphorylation capacity was similar to that of controls, as seen in the fluorescence intensities of reacting cells. Thereby PMNLs are capable of mediating tissue destruction in AP patients with immunosuppression.
**p38**

The p38 activation of the patients’ monocytes and PMNLs was similar to that of healthy controls, even though the patients were immunosuppressed. However, the proportion of pp38–positive lymphocytes was higher after stimulation with bacteria compared with controls, indicating that patients had an increased number of responding cells.

Activated p38 is a key player in the pathogenesis of inflammation (Schieven 2005), and in monocytes of the immunosuppressed patients, may play a critical role maintaining pro-inflammatory functions (Ayala et al 2000). In addition, MAPKs are associated with the development of systemic inflammation (Matsuda and Hattori 2006). In addition, activation of p38 is a prerequisite for fMPL–induced PMNL migration (Zu et al 1998). In Study III, patients’ PMNLs had higher transendothelial migration level than PMNLs from controls, suggesting that the patients’ PMNLs were able to maintain transmigration ability and PMNL-associated tissue destruction process despite severe immunosuppression.

**ERK1/2**

ERK1/2 activation of the patients’ monocytes, lymphocytes and PMNLs was depressed. To our knowledge, ERK1/2 activation in peripheral blood of AP patients has not been studied previously. ERK1/2 activation has an important role in chemotaxis (Wain et al 2002) and in transmigration (Serikov et al 2004). Indeed, in Study I transmigration of the patients’ monocytes was impaired, which may be explained, at least in part, by depressed ERK1/2 activation of the patients’ monocytes.

**STATs**

To our knowledge, STAT3 activation in peripheral blood of AP patients, have not been studied previously. In the present study, STAT3 was constitutively activated in lymphocytes in all of 13 patients studied. This finding was new. The partial activation of STAT3 was seen in CD3+CD4+ and in CD3+CD8+ T–lymphocytes, and could be completed in response to IL-6. In addition, constitutive STAT3 activation was seen in PMNLs of five
of 13 patients. Two of them died for multiple organ dysfunction syndrome. After stimulation with IL-6, the proportion of pSTAT6-positive PMNLs of the patients were higher than in controls. However, the phosphorylation levels of IL-6 stimulated PMNLs were comparable to that of controls.

Constitutive STAT3 phosphorylation has been reported to occur in cancer cells (Chen et al. 2008) and in lymphocytes of patients with systemic inflammatory disorders (Ramos H et al. 2008, Kuuliala et al. 2015). The constitutive activation of STAT3 in lymphocytes of AP patients with end organ dysfunction may originate from elevated circulating levels of IL-6 and IL-10 (Mentula et al. 2005). In addition, IL-6 together with IL-21 and IL-23 promote sustained STAT3 activation, which favor Th17 differentiation of CD4+ lymphocytes (Egwuagu 2009). It has also been shown that STAT3 may promote PMNL-mediated inflammatory tissue injury (Malchow et al. 2011), control PMNL migration (Nguyen-Jackson et al. 2010) and contribute to the development of acute lung injury (Severgnini et al. 2004). However, the pSTAT3 levels did not correlate with PMNL transendothelial migration.

STAT1 activation in both monocytes and lymphocytes in response to IL-6 was depressed in AP patients. Indeed, the phosphorylation levels, the proportion of pSTAT1-positive cells and the fluorescence intensities of IL-6 stimulated monocytes and lymphocytes all were lower in patients than in controls. This suggests that the defect involves all IL-6 stimulated monocytes and lymphocytes rather than a subset of them.

STAT1 is associated with pro-inflammatory signaling and development of Th1-mediated tissue injury and is activated by multiple cytokines (Brierley and Fish 2005). STAT6 is a mediator of Th2 signals and is associated with less injurious Th2-mediated tissue injury. STAT1 phosphorylation levels were lower and STAT6 phosphorylation levels were higher in patients’ lymphocytes than the respective phosphorylation levels in controls’ lymphocytes.

This implies that the STAT1/STAT6-ratio was depressed in the patients. These novel findings suggest that patients’ immune system tries to inhibit inflammatory tissue injury by reducing STAT1 activation by shifting tissue-destructive Th1-type immune response to a less injurious Th2-type immune response. Such a shift based on the STAT1/STAT6-ratio has been recently described in patients with rheumatoid arthritis (Kuuliala et al
a form of chronic inflammation of autoimmune type. The findings suggest that the method used suits for evaluation of the Th1/Th2 balance of the patients with systemic inflammation.

STAT4 phosphorylation in response to IL-4 was lower in the patients PMNLs than in controls' PMNLs. The overall effect of the depressed STAT4 signaling capacity on the patients' PMNL function is unclear because IL-4 may either activate (Lavoie-Lamoureux et al 2010) or inhibit (Marie et al 1996) PMNL effector functions.

3.4.2 Trypsinogen-3 (IV)

The immunoassay used in Study IV detects trypsinogen-3 with high specificity. The cross-reactivity with recombinant trypsinogens-1, -2 was less than 0.1% as defined by analysis of serum trypsinogen-1 and trypsinogen-2 fractions separated by ion exchange chromatography.

The present study revealed for the first time that serum concentrations of trypsinogen-3 in patients with AP are significantly increased, whereas the median concentration of trypsinogen-3 in healthy controls was less than 1.0 µg/l. Trypsinogen-3 represents only 4-10% of total trypsin in pancreatic juice (Rinderknecht et al 1979) but due to its ability to degrade PSTI (Szmola et al 2003) it may play a key role in the development of AP. Therefore, trypsinogen-3 may have a potential of diagnostic and prognostic value. Although the diagnosis of AP can be made from elevated concentrations of serum amylase (three times of upper reference limit), there was no relationship between amylase and trypsinogen-3 concentrations except for patients with alcohol-induced AP. This may reflect a different pathological mechanism for trypsinogen-3 and amylase.

According to the ROC –analysis, trypsinogen-3 discriminates AP patients from controls with the same high accuracy than amylase and trypsinogen-2. However, trypsinogen-3 did not have a predictive value for the severity assessment of AP.
3.4.3 Limitations of the study

Some limitations of the studies are worth mentioning.

Studies I-III were confined to a limited number of patients who had end organ dysfunction and whose clinical outcome and immune inflammatory status were reasonably comparable. We focused on these patients to screen for the affected signaling profiles in the most severe form of AP. All the patients in Studies I-III had AP complicated with persistent OD. Consequently, the patients had severe AP according to both previous (Bradley 1992) and current (Banks et al 2013) severity criteria.

The whole blood assay used in Studies I-III, does not permit to distinguish signaling defects that have their origins at the receptor level from those at the post-receptor level.

Meticulous sample handling is needed to avoid inappropriate cell activation *ex vivo*. Indeed, activation is known to occur even under simple stress conditions such as accelerated blood flow (Härtel et al 2001), density gradient centrifugation (Calafat et al 1993), changes in temperature (Kizaki et al 2001) and exposure to trace concentrations of LPS, an ubiquitous environmental contaminant (Haslett et al 1985).

Leucocyte permeabilization, which permits the phosphospecific antibody molecules to enter the cells, is a critical step in the whole blood assay. Indeed, a failure in sufficient permeabilization would prevent the phospho-specific antibody from entering into the cell, while extensive permeabilization might cause signaling protein leakage out of the cell. The permeabilization procedure used in Studies I-III permits the antibody to enter both cytoplasmic and nuclear compartments of the cell.

The possibility that the patients’ leucocytes are less permeabilized than leucocytes from controls, can not be excluded. Indeed, leucocyte activation may increase cellular resistance to membrane-active agents (Vuorte et al 2001). It is of note that the activation differences of NFκB between monocytes and PMNLs in one and the same sample may derive from the permeabilization differences between the cells.

The possibility cannot be excluded that some aberration(s) in signalling pathways associating with severe AP have been missed due to the small number of the patients studied.
The leucocyte signaling studies should be considered as a single center study, with strict inclusion criteria. Therefore generalization of the results should be done with caution. Indeed, most, but not all, of the patients with SAP show decreased HLA-DR level (Mentula et al 2003), which was the most important inclusion criterion. The aberrations in leucocyte signaling found in Studies I-III may be detected in some level in patients with SAP and normal HLA-DR, but there is no data available, however.

Despite the caveats above, the present results suggest that whole blood phospho-specific flow cytometry is a suitable method for immune monitoring of AP patients with systemic inflammation (McGregor and Mole 2010, Caldwell and Hotchkiss 2011).

Study IV consisted of 82 AP patients. Patients were classified by previous severity criteria as mild or severe AP (Bradley 1992). The current classification (Banks 2013) criteria could not be applied because clinical data were not available for distinguishing between transient and persistent OD. The aim of the study was to find out if the novel trypsinogen-3 assay is suitable for clinical use. As the first study to address this question, the number of the patients was satisfactory.

The delay from the sample collection to trypsinogen-3 measurement in Study IV may have influenced on the trypsinogen-3 levels. The samples were stored at -20° C for the median of 3.6 years before measurement of trypsinogen-3 concentrations. Trypsinogen-3 stability in serum samples was studied by assaying eleven serum samples from AP patients fresh and after a storage for median of 3.7 years at – 80° C. The recovered trypsinogen-3 concentration after the storage was 89% ± 23% (range 28-114%). The possibility that such a long storage time and freezing and thawing of the samples, have affected on trypsinogen-3 levels, is difficult to exclude with certainty on the present study.

3.4.4 Clinical and future aspects

The development of OD and secondary infections are the most important factors of morbidity and mortality in AP. Consequently, the most important clinical aspect of AP is to identify as early as possible the patients most likely to develop OD or secondary infection. In clinical practice, detecting the patients who present without OD but to
develop it during hospitalization is challenging. Indeed, half of the patients with AP complicated by OD do not have signs of OD at presentation (Johnson and Abu-Hilal 2001).

Secondary infections are usually seen at the late phase of SAP. The shift of the immune systems from a pro-inflammatory state to a compensatory anti-inflammatory state (CARS) may be excessive and lead to severe immune suppression (Bone 1996). The patients are susceptible to nosocomial sepsis or to development a local complication, such as infected necrosis, pseudocysts or abscesses (Banks et al 2013).

The present studies showed impairments of leucocyte signaling pathways in patients with AP complicated by OD and immunosuppression. These aberrations may offer a potential predictive marker for the development of OD and/or secondary infections. The results encourage to carry out prospective follow-up studies to find whether depressed NFκB and STAT1-activation capacity identifies patients at risk for secondary infections and whether increased p38 activation or depressed STAT3 activation identifies patients at risk for OD.

Finally, immune monitoring may disclose novel targets in the signaling pathways to alleviate the course of AP.

Trypsinogen-3 has a potential impact as a diagnostic tool in AP. However, the long storage time may have affected trypsinogen-3 levels, and therefore the findings needs to be confirmed by prospective studies with larger number of patients with different severities of the disease (Viljoen and Patrick 2011).
3.5 CONCLUSIONS

I) Peripheral blood monocytes of AP patients with OD showed impaired NFκB–, STAT1– and STAT3 –activation. ERK1/2 –activation was impaired, which may, at least in part, explain impaired monocytes transendothelial migration.

II) Peripheral blood lymphocytes of AP patients with OD showed impaired NFκB activation. p38 activation was enhanced. STAT3 showed constitutive activation, STAT1 activation was impaired and STAT6 activation was enhanced.

III) Peripheral blood polymorphonuclear leucocytes of AP patients OD showed decreased NFκB activation, while activation of p38 was normal.

IV) Serum trypsinogen-3 levels served as a diagnostic marker, but not as a marker of severity for AP.
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Kuopio, May 2017

Jani
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