Experimental septic shock – Effects of endotoxemia with special reference to pathophysiological responses in the pig

EWA SÖDERBERG

Sepsis and septic shock are conditions, with severe outcome or in many cases death. Sepsis is a systemic inflammatory response trigger by bacteraemia but systemic inflammatory response can also be triggered by major trauma, major surgery, pancreatitis, severe burns etc.

The systemic inflammatory reaction initiating the evolvement of septic organ dysfunction can be modelled using endotoxin, a Gram-negative bacterial lipopolysaccharide. This thesis used a porcine experimental sepsis model to examine timing of the inflammatory response due to endotoxin infusion (Paper I) and the influence of steroid treatment on the inflammatory response in endotoxemic pigs (Paper II). Timing of steroid treatment and the role of neutrophil granulocyte activation was evaluated with pig specific NGAL assessing neutrophil activation (Paper III). A clinical observational study was performed with the aim to differentiate between sepsis and other inflammatory conditions (e.g. trauma due to major surgery) evaluated by calprotectin as a marker of neutrophil activation (Paper IV).

There was a dose-dependency in endotoxin tolerance which was measured with TNF-a. Pre-exposure to endotoxin did not reduce the pulmonary response to endotoxemic challenge. In fact, both PaO₂ / FiO₂ and static pulmonary compliance were reduced in this group when pre-treated with endotoxin at low dose.

Endotoxemic animals treated with hydrocortisone were more stable in circulatory variables than those without such treatment. This was not explained by an ability of steroids to modulate the production of NO (Nitric oxide), which has been suggested to be a mechanism of steroids in this aspect.

Pre-treatment with hydrocortisone attenuated the neutrophil granulocyte response and consequently diminished the release of NGAL in plasma. Circulatory derangement was associated with high plasma NGAL levels. Urine NGAL levels did not differ among the four groups.

Plasma calprotectin levels on ICU admission is a sensitive marker of systemic inflammation and are markedly increased in patients with sepsis and patients with systemic inflammatory response. Plasma Calprotectin performed better than any of the other inflammatory variables in predicting mortality at 30 days, except from the composite mortality prediction score, SAPS 3.

**Keywords:** Endotoxin, inflammatory response, animal model, timing, steroid treatment, NGAL, Calprotectin

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Till mina älsklingar!
List of Papers:

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


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Abbreviations

AKI  Acute kidney injury
ALI  Acute lung injury
ARDS Adult respiratory distress syndrome
BE   Base excess
BSA  Body surface area
CAP37 Cationic antimicrobial protein, 37kDa
CI / CO Cardiac index / Cardiac output
CLP  Caecal ligation combined with puncture
COX  Cyclooxygenase
CRP  C-Reactive protein
CVP  Central venous pressure
DO2  Distribution of oxygen
ESR  Erythrocyte sedimentation rate
FiO2 Fraction of inhaled oxygen (%)
H    Hour
Hb   Hemoglobin
HBP  Heparin-Binding Protein
HNL  Human neutrophil Lipocalin
HR   Heart rate
GFR  Glomerular filtration rate (mL/min)
IBD  Inflammatory bowel disease
ICU  Intensive Care Unit
IL-6 Interleukin-6
kPa  Kilo Pascal
L1   Leucocyte 1 protein
LVSWI Left ventricular stroke work index
LPS  Lipopolysaccharide
MAP  Mean arterial pressure
MOF  Multiple organ failure
MPAP Mean pulmonary arterial pressure
MRP8 Myeloid-related protein 8
MRP14 Myeloid-related protein 14
NGAL Neutrophil gelatinase associated lipocalin
NO Nitric oxide
PaCO2 Arterial carbon dioxide tension
PaO2 Arterial oxygen tension
PCWP Pulmonary capillary wedge pressure
PEEP Positive end expiratory pressure
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ppeak</td>
<td>Peak proximal airway pressure</td>
</tr>
<tr>
<td>Ppause</td>
<td>Pause proximal airway pressure</td>
</tr>
<tr>
<td>PVRI</td>
<td>Peripheral vascular resistance index</td>
</tr>
<tr>
<td>RR</td>
<td>Respiratory rate</td>
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<tr>
<td>RRT</td>
<td>Renal replacement therapy</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SvO$_2$</td>
<td>Mixed venous oxygen saturation</td>
</tr>
<tr>
<td>SVRI</td>
<td>Systemic vascular resistance index</td>
</tr>
<tr>
<td>Temp</td>
<td>Temperature degrees Celsius</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TV</td>
<td>Tidal volume</td>
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<tr>
<td>VAP</td>
<td>Ventilator-associated pneumonia</td>
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1. Introduction

Sepsis is usually defined as the endogenous host response to invasive infection [1, 2]. Systemic illness caused by microbial invasion of normally sterile parts of the body is referred to as ‘sepsis’. The main purpose of this characterization is to differentiate ‘sepsis’ from systemic inflammation caused by mechanisms other than microbial invasion, e.g. pancreatitis. The clinical similarity between these two types of systemic inflammation may be explained by the fact that activation of the cytokine cascade is a fundamental event in both responses.

A rapid deterioration from sepsis to severe sepsis to septic shock is frequently seen, where the body’s inflammatory and anti-inflammatory responses evoke an inflammatory reaction that generates a multitude of reactions and events. When the responses to this potentially life-threatening state are associated with organ dysfunction and cardiovascular instability, the conditions are denoted ‘severe sepsis’ and ‘septic shock’, respectively. Despite early goal-directed therapy [3] and improvement in many aspects of supportive care, septic shock is still linked with an overall hospital mortality of approximately 30–50% [4, 5].

Loss of integrity in the barrier between sterile and non-sterile body compounds is a prerequisite for microbial invasion. Such disintegration may lead to penetration of pathogens through the skin or mucous membranes into the bloodstream. Microbial invasion may not only arise from obviously contaminated foreign bodies, which may be seen after trauma, but may also be caused by microbial growth on indwelling central venous catheters, endotracheal tubes, urinary catheters, or other devices. Furthermore, penetration is facilitated when the patient is immunologically compromised.

Clinical signs of septic shock and subsequent organ dysfunction include several features, e.g. confusion, oliguria, lactic acidosis, and/or SvO2 < 70%. Microbiological diagnosis is useful but not usually necessary for initial management. Samples for culture, microscopy and/or PCR should be taken before the start of antimicrobial therapy. Most frequently, identification of the causative microbial agents occurs after antibiotic treatment has been initiated [6].

Circulatory derangement is caused by a combination of vasodilatation, microvascular leakage, and reduced myocardial contractility. Catecholamines are used when i.v. fluid resuscitation is insufficient to restore adequate tissue perfusion. The quality of evidence on which to base the choice of therapeutic agents is relatively poor; the available literature does not show any difference.
in shock reversal, morbidity or mortality regardless of the vasoactive agent chosen [7-9]. Norepinephrine is commonly used as first-line agent. Its effect on blood pressure is usually prompt, thereby improving renal function. Norepinephrine causes only a modest rise in CO (cardiac output), but its effect on the liver and the gastrointestinal mucosa is unpredictable. Presently, non-catecholamine drugs, such as vasopressin, levosimendan, methylene blue and phosphodiesterase-inhibitors, have only a limited place in supporting the circulation of the septic patient. Septic shock is frequently accompanied by multiple organ failure (MOF). Pulmonary insufficiency is frequent and some patients develop ALI/ARDS (acute lung injury/acute respiratory distress syndrome). In addition, renal failure is frequently seen. Dialysis as well as hemofiltration have their place in the therapeutic arsenal.

Various types of severe sepsis affect approximately 0.5 – 1 million people annually in the U.S. alone [10], with the average cost per case being higher than $22,000 [4]. An investigation in a Swedish university hospital reported an overall mortality rate in sepsis of 25%, while the ICU mortality for patients with septic shock was almost 60% [11]. Despite guidelines from the Surviving Sepsis Campaign (SSC) and advanced medical care, severe sepsis and septic shock represent a global leading cause of potentially preventable morbidity or death [12].

Animal models of human pathology have both obvious advantages and disadvantages. Although animal experiments must not dictate human medical practice, they may be valuable in pointing out future therapeutic approaches for validation in clinical trials. Moreover, animal models give us a unique opportunity to study pathophysiological reactions and events in the absence of therapeutic interventions, in a way that would never be acceptable in humans. Endotoxin, live bacteria or faecal peritonitis are frequently used to initiate the inflammatory response and may hereby be used to evaluate the cascade of biochemical events that propagates as a response to this noxious stimulus. The focus is generally on septic shock, a disorder frequently seen in intensive care units worldwide that is characterized by an extensive inflammatory reaction and associated with high mortality.

This review concentrates on various animal models that mimic inflammatory conditions, especially the endotoxemic pig, as well as a clinical study comparing inflammation in different categories of patients admitted to the ICU.
2. Background

If the septic process is not effectively controlled by antimicrobial drugs and, when relevant, combined with surgical procedures, there is a risk for progression of the inflammation, that, in turn, may cause multiple organ failure (MOF). The number of failed organs correlates with mortality. Commonly, the sequence of organ failure is primary cardiovascular, followed by the lungs, kidneys and central nervous system. As MOF proceeds, the liver and the haemostatic system may also be involved [13]. The inflammatory response in severe sepsis and septic shock is complex and numerous interactions between pro-inflammatory and anti-inflammatory cytokines can be seen. As inflammation proceeds, anti-inflammatory mechanisms develop progressively [14].

The term ‘endotoxin tolerance’ is commonly attributed to a reduced responsiveness to a LPS (lipopolysaccharide) challenge following a first challenge with endotoxin [15]. Such tolerance ameliorates the consequences of a second hit of endotoxin and is followed by the up-regulation of inhibitory molecules that down-regulate the TLR (toll-like receptor) 4-dependent signalling pathway. Furthermore, endotoxin tolerance has been shown to reduce mortality and protect against organ dysfunction after ischemia-reperfusion injury [16]. The effect of endotoxin tolerance on sepsis manifestations, such as hypoperfusion and organ dysfunction after a second hit of endotoxin, has not been satisfactorily elucidated. In contrast to what one might expect, ARDS may be associated with endotoxin tolerance [17]. The endotoxemic pig offers an exclusive possibility to study the pathophysiological reactions and events associated with endotoxin tolerance.

Steroids in sepsis

Whether steroids should or should not be used in septic shock remains unclear, although numerous clinical trials have aimed to affirm or disprove such treatment. Nevertheless, high-dose treatment with steroids has no place in the modern treatment of septic shock [18, 19]. The possible benefits of a low-dose regimen are however, not, ruled out since a meta-analysis showed that low-dose steroid treatment appeared to reduce mortality rates in patients with septic shock [20]. Even though, such treatment may cause steroid-induced side effects such as super infections, bleeding and hyperglycaemia.
The CORTICUS trial did not reveal any difference in survival between hydrocortisone-treated groups and placebo-treated groups, even though the time-to-shock reversal in the hydrocortisone group was significantly shorter [18]. Nevertheless, according to a meta-analysis, a daily dose of 200 to 300 mg hydrocortisone (or equivalent) may be beneficial in adults with vasopressor-dependent septic shock [21].

The timing of corticosteroid therapy seems to be crucial, since administration of steroids within 6 h (hours) after the onset of sepsis-mediated persistent arterial hypotension significantly reduced 28-day mortality as compared to corticosteroid therapy initiated after 6 h of hypotension (32% vs. 51%, \( P = 0.01 \)) [22]. Although these findings speak in favour of early onset of steroid therapy, it must be borne in mind that these findings were obtained retrospectively [22].

Glucocorticoids may also interfere with the cytokine cascade. Steroids administered together with or shortly before i.v. endotoxin to human volunteers prevented increase in TNF [23]. However, when glucocorticoids were administered 12 h or more before endotoxin challenge, the rise in TNF was not blunted. In a clinical study, hydrocortisone administered to septic shock patients requiring inotropic support was followed by improvements in SOFA (Sepsis Related Organ Failure Assessment) score and reduced plasma levels of IL-6, but not in TNF [24]. The effect of hydrocortisone on biomarkers in established endotoxemic shock is still not clarified.

Differentiating between sepsis and inflammation

Sepsis is a condition associated with severe morbidity and mortality. Inflammation, sometimes termed systemic inflammatory response syndrome (SIRS), is often one of the initial symptoms of sepsis as well as other conditions, e.g. major trauma, major surgery, pancreatitis and severe burns.

Searching for a biomarker that can differentiate sepsis from systemic inflammation has been going on for many years. Patients with systemic infection, often in septic shock, are in a critical condition and every minute counts. It is therefore not possible to await cultures or other time-consuming analyses before treatment. The lack of specific early biomarkers of infection may in part be responsible for withholding or delaying the treatment in critically ill patients or unnecessary antibiotic treatment. A biomarker, ideally used as a bedside test, would be the perfect solution for managing patients in this situation.

The Longitude Prize is one example of the importance of early diagnosis and choice of therapy. Run by Nesta and supported by Innovate UK as funding
partner, this is a challenge with a £10 million prize to reward a diagnostic test that helps solve the problem of global antibiotic resistance.

2.1 Animal models

Although the physiology of humans and animals is similar, research utilizing animal models has not always given results that are applicable in humans. On the other hand, one cannot defer from using animal models for sepsis research, even though clinical research has a limited ability to explain pathophysiological phenomenon. Response to pathologic processes may vary between humans and the animal models due to biologic differences and this could yield misleading conclusions about human pathophysiology. For instance, sensitivity to endotoxin, commonly used to elicit sepsis-like symptoms, varies considerably between different species; primates especially humans, are several magnitudes more sensitive to endotoxin than pigs, dogs and rats [25]. Furthermore, whereas the common patient with septic shock is an aged person with multiple systemic diseases, most research animals are young and healthy. Most importantly, many sepsis models study animals in early disease stages and mortality within a few hours as the endpoint, while in the clinical setting, patient mortality is seldom as close in time to the first signs of inflammation [26].

Nevertheless, animal models provide a most relevant tool for studying several pathophysiological phenomena during sepsis. Early inflammatory mediators, for example, have been strongly conserved throughout evolution. Endotoxin administered to animals or humans induces many features of sepsis such as cytokine release, leucopenia, fever, cardiovascular changes, nephropathy and coagulopathy. Administration of endotoxin to human volunteers has been used in several studies, although it must be remembered that such experiments always carry a risk of unforeseen, potentially life-threatening complications [27]. The possibility of performing standardized, extensively monitored animal experiments, in combination with the opportunity to administer a broad spectrum of dosages of endotoxin, gives the option of evaluating pathophysiological events and reactions that ultimately result in death, with a high degree of reproducibility.

Thus, results from animal models still represent a major source of knowledge in medical research. It should, however, be kept in mind that these are just models and that the findings in animal experiments are merely the basis for further investigations in humans.
2.1.1 Animal species

The choice of laboratory animal species is determined by the organ systems to be studied, local legislation, project budget, number of animals needed, housing possibilities, animal size and local traditions with existing models. Considerable knowledge has accumulated on sepsis models in different animal species.

Despite being the most frequently used laboratory animals, there are some experiments that cannot be performed on mice and rats. Even though acute inflammatory stress from different aetiologies, i.e. trauma, burns and endotoxemia, results in highly similar genomic responses in humans, the responses in corresponding mouse models correlate poorly with the human condition and also with one another [28]. Relative to the human response, mice are highly resilient to inflammatory challenge. For example, the lethal dose of endotoxin is a million-fold higher in mice vs humans [29]. However, small rodents (mice and rats) are particularly suitable for models where long-term observation or a high number of animals are required.

Larger rodents, e.g. rabbits, are less commonly used in inflammatory research. Nevertheless, rabbits offer some advantages. Their blood volume is much larger, they are suitable for antibody production, and are useful for experiments that require large volume sampling. However, the size of rabbits still limits the use of advanced circulatory and respiratory equipment.

The dog is today a popular pet, which limits its use for ethical reasons. Even so, dogs are still used for certain models since they are easy to handle and are large enough for surgical instrumentation and cardio-respiratory monitoring.

Pigs have many of the advantages that dogs offer and are often easy to obtain from local producers, without long stressful transportation. The juvenile pig is large enough for instrumentation and the use of human medical equipment. In addition, the blood volume of the juvenile pigs allows extensive blood sampling, making it suitable for intensive care models. Porcine endotoxin shock models have been widely employed in experimental sepsis research. Although the circulation of the pig has been shown to be most similar to that of humans among non-primates [25, 30], its lung vascular smooth muscle is very sensitive to live bacteria and endotoxin, which leads to a substantial increase in pulmonary vascular resistance. Increased pulmonary vascular resistance leads to hypodynamic circulation in porcine sepsis models, unlike the hyper dynamic circulation seen in human septic shock.
As can be expected, the closest resemblance to human septic response is seen in primates. Experiments in baboons have contributed to unveiling important immunological mechanisms related to the development of organ dysfunction [31]. But despite the fact that these animals are biologically ideal, their models tend to be less and less used. Many species are endangered, legislation restricts or forbids their use, and even non-endangered species are costly.

The choice of research animals has to take a great number of factors into account, any species used will lead to compromises. Apart from the animal’s species, their sex [32], age [33], strain [34] and nutritional status [35] may all influence the model and might lead to experimental variation.

2.1.2 Sepsis models

Sepsis models have been based on the observation that sepsis induces a generalized inflammatory response commonly referred to as SIRS (systemic inflammatory response syndrome) [1]. Following the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3), 2016, the SIRS concept has been abandoned and sepsis is now defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection. Organ dysfunction can be identified as an acute change in total SOFA (the Sequential [Sepsis-related] Organ Failure Assessment) score of two points consequent to the infection [36-38]. However, the systemic inflammatory response is still a useful concept.

Irrespective of cause, once systemic inflammatory response is established, the pathophysiological mechanisms, i.e., activating the innate and adaptive immune system, are similar. However, some variations exist in SIRS induced by different causes.

One problem with the concept of sepsis and SIRS definitions in experimental research is that criteria for these conditions (tachycardia, tachypnoea, hypo- or hyperthermia and leucocytosis or leucopenia and bacteraemia) cannot be applied in most animal models. Heart rate, breathing frequency, core temperature and white cell counts usually differ from human reference intervals. Nevertheless, the experimental setup and changes from baseline levels in these variables indicate the validity of sepsis models in animals.

Most sepsis models induce a sepsis-like systemic inflammatory syndrome. Depending on the model, some aspects of the septic syndrome will be present or absent and this has to be taken into consideration when designing a study. Several different animal models have been utilized to experimentally mimic...
human septic shock. For example, the most commonly used methods, endotoxemic shock and CLP (caecal ligation combined with puncture), have significant differences in the kinetics and magnitude of cytokine production [39]. Apparently, these regimens also differ regarding the arachidonic acid cascade, since COX-2 inhibition improves early survival in endotoxaemic mice, but not in those with CLP [40].

The three main types of sepsis models are the endotoxemic, the bacterial and the peritonitis.

2.1.3 Endotoxemic models

Sensitivity to endotoxin is a phylogenetically-conserved ‘alarm system’ that functions as an activator of the immune system if Gram-negative bacteria are present. These bacteria are potent immunostimulators in eukaryotes - from the small two-winged fruit flies used in genetic research (Drosophila melanogaster) to Homo sapiens [41-49]. This property has been extensively utilized in sepsis models where endotoxins are used to trigger the inflammatory response seen in sepsis in order to replicate human Gram-negative septic shock.

The terms endotoxin and lipopolysaccharide (LPS) are used synonymously, although the former is usually used in the context of biological activity while the latter is used to describe the macromolecule. Endotoxin comprises a toxic lipopolysaccharide moiety that Gram-negative bacteria carry on their surface. The LPS layer of the outer membrane of Gram-negative bacteria forms an effective permeability barrier against external stress factors. This membrane is permeable only to low molecular weight, hydrophilic molecules, which renders it a barrier to, for example, lysozyme, bile acids and many antimicrobial agents. LPS is a group of compounds with varying biological activity. The polysaccharide and the lipid portion of LPS contribute to the pathogenic potential of this class of bacteria, where the lipid component (lipid A) determines the endotoxic properties of LPS.

As LPS can relatively easily be stored, quantified, and administered in a standardized dose, it represents a simple and reproducible model. It may be administered either as a continuous intravenous infusion [50] or as a bolus intravenously [51], intraperitoneally [52], or intratracheally [53].

Whereas endotoxin elicits a hyperdynamic cardiovascular response in humans, the pattern of response is characteristically biphasic in large mammals such as the pig. After the start of continuous endotoxin administration, the cardiac output decreases with its lowest point after about three hours. By eight to twelve hours, it reaches supra normal values.
Hyperdynamic porcine models are characterized by long observation periods with the first cardiac output measurements after six hours. Long observation periods are cumbersome since long-term anaesthesia or sedation may influence findings, whereas the preservation of intravenous access in non-anesthetized animals may be problematic. Furthermore, animal welfare regulations, based on ethical considerations, restrict the use of awake-animals in experiments where considerable suffering is expected. This results in observation periods that are usually less than eight hours into experimental sepsis. In order to reach the state of multi-organ failure, relatively high endotoxin doses are used, which may be rare in clinical sepsis. This may produce a condition of acute intoxication rather than a gradually developing sepsis. One other consequence of these rather short observation periods is that these models are more suitable for investigating the phenomena in the early pro-inflammatory phase of sepsis [54].

Endotoxin models are also of interest when drugs or other treatments with a proposed anti-endotoxin effect are being investigated [55-57]. Since bacterial factors other than endotoxin also may contribute to the host response, animal models using infusion of live Gram-negative bacteria [58], usually E. coli (Escherichia coli), sometimes have been proposed to be more clinically relevant than endotoxin models [59]. Nevertheless, the pathophysiological changes in live bacteria models are more or less similar to the endotoxin infusion models.

2.1.4 Live bacteria models

Given that the inflammatory reaction in sepsis is due to microbial presence in the circulation, intravenous infusion of live bacteria would seem to be the method of choice for sepsis models. However, since live bacteria require facilities where they can be safely kept, cultured, typed and quantified, these models are more troublesome than most others.

The strain of bacteria used in the model depends on the clinical condition to be studied. One should remember that within a bacterial strain, the subtype may also have a substantial impact on the experimental model. For example, over 700 serotypes of E. coli with varying virulence have been described. A highly virulent E. coli serotype may be given in a low dose to correspond to the desired clinical situation to be modelled. On the other hand, bacteria with low virulence may be easier to dose and quantify since they need to be administered in a higher dose.
In short duration experiments where bacteria are given intravenously, the bacterial growth cycle should also be considered. For these experiments, bacteria should ideally be in log-phase i.e. dividing, when administered to the laboratory animals. Apart from intravenous infusion [31, 60, 61], intraperitoneal [62], intratracheal [61] and subcutaneous administrations [63] are possible. If a microbiological laboratory is not available, the mixed flora of peritonitis models, described in the next section, offer a clinically valid model.

2.1.5 Peritonitis models

These models resemble human abdominal sepsis with a focal abdominal infection developing into septic shock. The three most common types of peritonitis sepsis models are CLP (caecal ligation puncture), colon ascendens stent peritonitis, and peritoneal faeces implantation models. The two former are usually performed in small rodents. A principally important property of these models is that sepsis develops relatively slowly from a focal to a systemic inflammatory process, mimicking the clinical situation. Furthermore, these models are relatively simple to perform and are reproducible, which makes relatively long observation times possible. Being rodent models, the cost per animal is also relatively low. Drawbacks are mainly due to the relatively small animals limiting blood sampling, difficulties in monitoring pathophysiological changes, and difficulties in performing various interventions.

As discussed earlier, biological differences in rodent and human physiology may complicate the interpretation of results in the context of human sepsis.
Several biomarkers of inflammation are used in daily medical care. Unfortunately, none of them are so far able to discriminate between bacterial, fungal and viral infections, nor between inflammation due to trauma, caused by major surgery or as a response to severe infection. The possibility to differentiate between the origins of an infection as a cause of inflammatory response would be very useful when it comes to treating severe infections that cause sepsis as well as for choosing among the potential therapies available.

One of the most common biomarkers used in all levels of medical care is CRP (C-reactive protein). Discovered over 70 years ago by Tillet and Francis [64], CRP is an acute-phase protein in humans, produced in the liver in response of IL-6. Its synthesis is synergistically enhanced by IL-1ß via the NF-κB receptors p50 and p65 [65-67]. CRP, a member of the pentraxin protein family, consists of a ring of five identical non-covalently bound subunits [68]. It has been used clinically to detect acute infections and to assess the response to treatment. A very important property of CRP is its ability to activate the classical complement cascade that in turn is a factor in the killing of microorganisms and mediating protection from pathogenic bacteria [68]. Serum levels of CRP are also used to distinguish between bacterial and viral infections, since the former generally cause a more pronounced elevation of CRP levels [69].

ESR (erythrocyte sedimentation rate) is the rate at which red blood cells sediment in one hour. The test was developed by the Polish pathologist E. Biernacki in 1897. ESR is still called Biernacki’s reaction in some parts of the world [70]. In 1918 the Swedish pathologist R. Fähræus, together with A. Westergren, refined the test with sodium citrate-anticoagulated specimens and since then it is also called the F.W. (Fähræus-Westergren) test or just the Westergren test [71]. ESR is a non-specific test of inflammation performed with anti-coagulated blood in an upright tube; the Westergren tube. The rate at which the red blood cells fall is measured and reported in mm/h. The test is still used in diagnosing some diseases, such as multiple myeloma, temporal arteritis and polymyalgia rheumatica, in which the ESR may well exceed 100mm/h [72]. Patients with Kawasaki’s disease have markedly elevated ESR and the test is commonly used in these patients for differential diagnosis [73].

Procalcitonin is a glycoprotein and the pro-peptide of calcitonin devoid of hormonal activity. It consists of 116 amino acids, has a molecular weight of
13kDa, and is under normal circumstances produced in the C-cells of the thyroid gland. Its half-life in serum is 25-30 h [74]. In healthy humans, Procalcitonin is undetectable (<0.1ng x mL$^{-1}$) but during severe infections, e.g. sepsis or septic shock, levels may rise to >100ng x mL$^{-1}$. This Procalcitonin is mainly produced by extra-thyroidal tissues [75]. Markedly increased inflammation (acute pancreatitis, severe burns, chemical pneumonitis etc.), may per se, also result in increased levels of Procalcitonin [76].

Heparin-Binding Protein (HBP), (also known as azurocidin or CAP37, a cationic antimicrobial protein of molecular weight 37kDa) is stored in the azurophilic granules in the neutrophils. HBP is released upon neutrophil adhesion to endothelial cells. The molecular mechanisms of HBP, which function as an inflammatory mediator causing uncontrolled increase of vascular permeability, including capillary endothelial leakage with extravasation of plasma and white blood cells (WBC) to the infection site, are of key importance in sepsis [77]. HBP has been proposed as a predictor for vascular failure in sepsis [78]. A high plasma concentrations of HBP on admission to the ICU is associated with respiratory failure, circulatory failure and mortality, but its value as a biomarker in the individual patient in an ICU with a variety of diagnoses is low [79].

NGAL is a small protein with a molecular weight of 25kDa and a short duration half-life of 10-20 minutes [80]. NGAL, also known as Lipocalin 2 or HNL (human neutrophil Lipocalin), was originally identified as a protein released following neutrophil activation [81-83]. It is stored in the granules of the neutrophil granulocytes, which comprise different forms, e.g. peroxidase-positive granules (azurophil) and specific peroxidase-negative granules (peroxidase-negative-lactoferrin positive granules (16%), granules containing both lactoferrin and gelatinase (60%) and granules containing solely gelatinase, so-called gelatinase granules (24%)). Gelatinase granules are less dense and smaller than the other peroxidase-negative granules. NGAL is localized in the granules containing both lactoferrin and gelatinase [81, 84].

NGAL appears early in the granulocyte formation and is a marker of neutrophil formation [85]. The primary bacteriostatic function of NGAL resides in its binding and confiscation of bacterial siderophores which deprives bacteria of iron [86]. Released NGAL not only has bacteriostatic effects, it is also important for maturation and activation of neutrophil leucocytes [87]. NGAL also stimulates neutrophil adhesion and extravasation, and acts as a chemoattractant together with lactoferrin [87, 88]. Although mainly released by neutrophil granulocytes, NGAL is present in the epithelial cells of several organs (colon, uterus, trachea, ventricle, prostate and salivary glands) as well as in the hepatocytes of the liver, in the tubulus of the kidneys, and in type II pneumocytes of the lungs [89].

NGAL, among many other biomarkers, has attracted interest as a biomarker of inflammation and acute kidney injury (AKI). NGAL can be quantified in urine and in cases of AKI, a considerable increase can be detected already after 2-6 h [90]. Open cardiac surgery, which must be regarded as a major
trauma with substantial inflammatory response, results in significantly elevated levels of NGAL already after 2 hours [91].

In a recently published article, the majority of patients with multi-organ dysfunction syndrome (MODS) and septic shock-related deaths had elevated plasma levels of NGAL at time of death [92]. High levels of plasma NGAL were found to be an independent factor for predicting MODS and mortality in patients with severe sepsis and septic shock, even after adjustments for confounding risk factors [92].

The inflammatory response of the body reacts very quickly when challenged with an infusion of endotoxin, and one of its promptest reactions is the release of NGAL from neutrophil granules via the TLR4 (toll-like receptor 4) [93]. The release of NGAL leads to a multitude of different cytokines erupting into the circulation, which in turn affects different organ systems, i.e. the coagulation system, and the migration of new neutrophil leucocytes to their targeted locations.

NGAL, as well as other biomarkers, has been evaluated in different trials trying to find a biomarker that can discriminate between virus and bacterial infections. The result of these studies have, so far, not been successful [94].

Calprotectin is a calcium-binding protein found in the neutrophil granulocytes where it comprises up to 60% of the cytosolic protein content of the neutrophils. It is a member of the S-100 family of calcium-binding proteins [95, 96] and is described in the literature under several different synonyms (complex of S100A8 and S100A9 proteins, 27E10 antigen, myeloid-related protein 8 and myeloid-related protein 14 [MRP8/14], Leucocyte 1 light protein (L1L) and Leucocyte 1 heavy protein (L1H) proteins and calgranulin A/B). Calprotectin is a heterodimer composed of light (MRP8) and heavy (MRP14) chains (8 and 14 kDa) [97, 98]. It contains zinc-binding domains having a higher zinc-binding capacity than other S100 proteins and is not affected by the binding of calcium. Both MRP8 and MRP14 contain histidine-based zinc-binding sequences that are involved in calprotectin’s antibacterial activities [99].

Calprotectin has been found to have clinical relevance in rheumatoid arthritis, IBD (inflammatory bowel disease), colorectal cancer, HIV, and other inflammatory diseases [100]. Faecal Calprotectin correlates well with endoscopic and histological grading of disease activity in ulcerative colitis [101] and reflects the degree of inflammation in remission or before diagnosis in IBD [102]. Elevated levels of Calprotectin in patients with IBD are a more sensitive and reliable marker than standard inflammatory biomarkers (e.g. ESR, CRP) [102].

Serum Calprotectin has been used to follow disease activity in rheumatoid arthritis. In patients receiving TNF-inhibitors, serum Calprotectin levels correlate better than CRP or ESR and are more reliable in patients with low inflammatory activity [103].
Regardless of age, neonatal sepsis, like severe sepsis and septic shock, is an extremely severe condition where acute phase proteins with a prompt reaction would be of considerable value. Results for serum Calprotectin in neonatal sepsis have showed promise and it may be considered as an early biomarker for neonatal sepsis [104].
4. Inflammatory responses and porcine endotoxemia

We have previously shown that the endotoxemic porcine model is well suited for evaluating various inflammatory responses secondary to endotoxemic challenge [105]. Several, but not all, of these responses were dose-dependent, which may be a useful guide when future experiments are designed, as well as when results are interpreted. Not only the administered amount of endotoxin is of importance for the host response, but also the administration rate [106]. A rapid increase of administered endotoxin elicits a more pronounced reaction in several variables, in contrast to a more modest step-wise increase. Interestingly, the cytokine network responds differently to different administration rates of endotoxin, even though the totally administered amount of endotoxin was the same in both groups. An increase in TNF-alpha, but not in IL-6, was more apparent when endotoxin was administered at a higher rate. Attempts have also been made to reduce the circulating levels of endotoxin in order to blunt some clinical manifestations of this challenge. We designed another experiment to focus on the effect of considerably reducing endotoxin load during the later phase of the experimental period [107]. Such early endotoxin elimination caused an extensive reduction in the circulating levels of endotoxin, but had essentially no effect on TNF-alpha-mediated toxicity. A minor effect on leukocyte response was associated with limited improvements in respiratory function and microcirculation. These effects were minor in relation to the magnitude of the endotoxin concentration reduction. Since the combination of aminoglycosides and beta-lactam antibiotics offers efficient therapy in conjunction with low antibiotic-induced endotoxin release, we decided to evaluate whether aminoglycosides aggravate sepsis-induced acute kidney injury [108]. Such a study cannot be performed in man, but the endotoxemic pig offers an excellent model for such a trial. Although the short duration of the study is a limiting factor, it does not strengthen the postulate that a single dose of tobramycin aggravates endotoxin-mediated renal damage.

Since oxidative stress and enzymatic lipid peroxidation are crucial events in the inflammatory cascade, an overview of their molecular biology is presented: nitric oxide (NO), originally described as an endothelial-derived relaxing factor, has been shown to be of uttermost importance as an intercellular messenger, regulating a huge variety of fundamental cellular functions, and
also deeply involved in mediating cellular damage in a wide range of conditions. NO is important as a toxic defence molecule against infectious organisms. Furthermore, recent research suggests that most of the cytotoxicity ascribed to NO is more likely due to peroxynitrite (ONOO\(^-\)) synthesized from the diffusion-controlled reaction between NO and another free radical, the superoxide anion (O\(_2^-\)), which interacts with various tissues, as well as with DNA and proteins. NO is an intermediate between molecular oxygen and nitrogen. All of these three molecules (NO, O\(_2\) and N) pass easily through membranes and cytoplasm. Molecular oxygen has two unpaired electrons, which allow it to bind strongly with metals such as the iron in hemoglobin and also cytochrome-c oxidase. It has the capacity to react instantly with the unpaired electron of other free radicals, where the second unpaired electron is in a reactive state. Oxidative stress is the net result of molecular oxygen facilitating free radical damage through these mechanisms [109]. It has profound effects during human septic shock, and xanthine oxidase, a marker of such damage, was increased in non-surviving patients, which could be used to predict outcome [110].

To summarize, in order to improve our understanding of pathophysiological mechanisms and new treatment options, we need standardized conditions where we do not have to intervene with the natural cause of the disorder in question. Thus, we need to be able to perform experiments where science and the quest for knowledge instead of medical practice is the primary focus. To achieve this, animal models still have a place in the scientific paradigm. We have adopted a model of porcine endotoxemic shock in order to mimic human septic Gram-negative shock. This model is extensively described and several other animal models are reviewed. Various methods used to induce inflammatory conditions are also described and assessed.

Sepsis is a common and in many instances deadly condition in intensive care [111, 112], presenting with systemic inflammatory response syndrome (SIRS). However, several other conditions also present with SIRS, such as post-operative state after major surgery, trauma, pancreatitis and burns [1, 113]. This makes early identification of patients with sepsis challenging in many instances. Since early initiation of correct antibiotic therapy has substantial impact on outcome, [12, 114] and antibiotic treatment in non-infectious causes of SIRS is of little benefit, [115] differentiating sepsis from other non-infectious causes SIRS is of major importance.

With the aim of searching for such a biomarker, we decided to transit into the clinical reality and investigate patients in the ICU. We have compared patients with septic shock with patients exposed to major surgery and with intoxicated patients.
5. Aims of my thesis

The aims of this thesis were:

- To study whether sepsis manifestations are equally affected under standardized intensive care therapy in a pig model in which the animals were exposed to a second hit of endotoxin after a 24-h low-dose endotoxin infusion compared with the responses in animals unexposed to endotoxin and intensive care, and to propose a model of intensive care secondary sepsis.

- To explore whether endotoxin tolerance is mainly an on-off phenomenon by studying the dose dependency of two pre-exposure and two challenge doses.

- To prospectively evaluate the effects of hydrocortisone, administered at a clinically relevant dose, on central hemodynamics and pro-inflammatory cytokines TNF-α (tumour necrosis factor alpha) and IL-6 (interleukin 6), when given at the onset of porcine endotoxemic shock, defined as doubling of MPAP (mean pulmonary arterial pressure), compared to the baseline value.

- To evaluate whether the timing of administered hydrocortisone has an impact on the neutrophil activation and subsequently the release of NGAL (neutrophil gelatinase associated lipocalin) in plasma and urine, when administered at a clinically relevant dose, before and at the onset of porcine endotoxemic shock.

- To describe the difference in central hemodynamics between the four research groups.

- To investigate whether plasma Calprotectin can differentiate patients with sepsis, i.e. patients with infection-triggered SIRS, from post-operative patients, i.e. SIRS triggered by major surgery, and from ICU patients with intoxication, i.e. patient without SIRS.

- The secondary goal was to investigate value of plasma Calprotectin in the predicting 30 days’ mortality.
6. Materials and methods

6.1.1 Anaesthesia, preparation and maintenance of vital functions in the animal models

A brief and somewhat standardized description of our experience, partly from a practical point of view regarding the endotoxemic pig model, is given below. Inclusion criteria are: baseline arterial oxygen tension (PaO$_2$) $\geq$ 10 kPa (75 mm Hg) and a mean pulmonary arterial pressure (MPAP) $<$ 2.7 kPa (20 mm Hg) 30 minutes after completion of the surgical procedure. Both criteria are sensitive markers of respiratory or systemic infection and are thus used as a screening method in order to minimize unnecessary variation in the acquired data. Animals of both sexes are used. The pigs commonly weigh between 20 kg and 30 kg. Larger animals have lower frequency of infections and have gone through a significant degree of selection but pose higher demands in terms of research facilities, instrumentation and drug expenses.

We gave each pig an intramuscular injection in the nuchal region of 6 mg x kg$^{-1}$ of tiletamin-zolazepam mixed with 2.2 mg x kg$^{-1}$ of xylazin in order to induce anesthesia while still in its cage. The animals do not seem to react to this stimulus and they usually fall asleep within a few minutes.

Although the animal’s airway usually remains patent and the respiratory drive is sustained, care should be taken to avoid asphyxia during this period. An intravenous cannula is easily inserted into a vein into the ear of the sleeping pig upon which 20 mg of Morphine and/or 100 mg of Ketamine is administered to enhance the level of anesthesia. A tracheotomy is easily performed in order to secure the airway. This can be performed through a midline incision between the cricoid cartilage and the area corresponding to the supra sternal notch. Blunt dissection strictly in the midline allows the trachea to be intubated through a semi-circumferential incision between two tracheal cartilages. This whole procedure can be performed in one minute. A tracheotomy in the pig is much easier to perform than in man.
When the airway is secured, a continuous intravenous infusion of 2.5% glucose with electrolytes containing sodium pentobarbital (8 mg x kg\(^{-1}\) x h\(^{-1}\)), pancuronium bromide (0.26 mg x kg\(^{-1}\) x h\(^{-1}\)) or rocuronium bromide (0.5 mg x kg\(^{-1}\) x h\(^{-1}\)) and morphine (0.48 mg x kg\(^{-1}\) x h\(^{-1}\)) was started in order to maintain the anesthesia. Infusion rate is 8 mL x kg\(^{-1}\) x h\(^{-1}\) with an additional amount of saline resulting in a fluid administration rate of 10-30 mL x kg\(^{-1}\) x h\(^{-1}\), depending on the experiment. Initial respiratory settings were as follows: TV (tidal volume), 10 mL x kg\(^{-1}\), FiO\(_2\) (inspired oxygen fraction) 0.3, RR (respiratory rate) to 25 breaths per minute and a PEEP (positive end-expiratory pressure) of 5 cmH\(_2\)O. The goal was to obtain a PaCO\(_2\) of between 5.0 kPa and 5.5 kPa. At the hourly measurements, ventilation was adjusted when needed according to the protocol.

A central venous line and a 7 F Swan-Ganz catheter equipped with a thermistor were inserted through the external jugular vein into the superior caval vein and the pulmonary artery, respectively. It should be remembered that the distance from the insertion in the vessel to the pulmonary artery is much shorter than in man. The vein can be found a few centimetres laterally from the midline. We inserted the vascular catheters surgically. The external jugular vein is thus divided and ligated. Below the divided vein, a cervical artery can be localized and catheterized for pressure monitoring and blood sampling. Since it is not possible to introduce a urinary catheter into the bladder through the urethra, we suggest that a urinary catheter is inserted through a small vesicostomy. The animals were kept warm by a heating pad and, when needed, covered with a blanket. After induction of anesthesia and surgical preparation, each animal was placed in the prone position or on the side, changing position every third hour, in order to prevent atelectasis.

Endotoxemia is induced by a continuous infusion of E. coli endotoxin (O111:B4; Sigma Chemical, St. Louis, MO, USA) at a dose of 0.063-4.0 µg x kg\(^{-1}\) x h\(^{-1}\). Doubling of the initial MPAP during the first hour of the endotoxin infusion is frequently accepted as a sign of endotoxemic shock. If the mean arterial pressure equals the same level as the mean arterial pulmonary pressure within the first hour after baseline, a single dose of 0.1 mg adrenalin or 20 µg noradrenalin was administered intravenously. The animals that survive the experimental period are killed by an intravenous overdose of potassium chloride monitored by electrocardiogram while still under anesthesia, which is confirmed with a mechanical-physical termination according to the local ethical guidelines.

6.1.2 Observational study in the ICU

This clinical observational study was performed at the general ICU of Uppsala University Hospital (Sweden). The ICU is located in a tertiary university hos-
pital with 900 beds. This is a nine-bed mixed ICU with both medical and surgical patients and approximately 1000 patients are admitted per year. All patients were treated according to medical science and proven experience.

We collected data on laboratory parameters from blood samples taken on admission, vital signs, ventilator and vasoactive treatment and Simplified Acute Physiology Score 3 (SAPS 3) [16].

6.1.3 Objective of Papers I-IV

In this experimental pig model (Papers I-III), we examined the timing of the inflammatory response due to endotoxin infusion (Paper I) and the influence of steroid treatment on the attenuation of the inflammatory response in endotoxemic pigs (Paper II). The timing of steroid treatment and the reaction of the innate immune system were evaluated with pig-specific NGAL assessing neutrophil activation (Paper III).

As treatment of humans is the purpose of our work, we performed a clinical observation study aiming to differentiate between human sepsis and other inflammatory conditions (e.g. trauma due to major surgery) evaluated with calprotectin as a marker of neutrophil activation (Paper IV).

6.2 Analysis

A commercial porcine-specific sandwich enzyme-linked immunosorbent assay was used for the determination of TNF-alpha (tumour necrosis alpha) and IL-6 (interleukin 6) in plasma (DY686 and DY690; R&D Systems, Minneapolis, Minn). Leukocyte count was analyzed on a CELL-DYN 4000 hematology analyzer (Abbott Scandinavia AB, Kista, Sweden). Creatinine measurements were analyzed on an Architect Ci8200 analyzer (Abbott Laboratories, Abbott Park, IL). Blood cell count and hemoglobin were analyzed on a CELL-DYN 4000 (Abbott, Abbots Park, IL) and plasma creatinine (reagent: 14.3600.01, Synermed International, Westfield, IN) was analyzed on an Architect Ci8200® analyzer (Abbot, Abbott Park, IL). Creatinine clearance was calculated as urinary creatinine x hourly diuresis/plasma creatinine x 60. Hydrocortisone in plasma and urine was analyzed on a Modular E170 (Roche Diagnostics, Mannheim, Germany) with reagents from the same manufacturer. The total analytical imprecision of the assay was 2.5% at 114 nmol/L.
and 1.7% at 672 nmol/L. Arterial and mixed venous blood gases were analyzed hourly by a ABL™ 300 and Hemoximeter™, Radiometer, Brønhøj, Denmark. Lactate was analyzed by the i-STAT® System (Abbott Point of Care, Princeton, NJ). Total nitrate/nitrite and nitrite was measured with Parameter™ kit after deproteinising the samples (KGE001, R&D Systems, Minneapolis, MN). Samples were analyzed for nitrite and for total nitrite/nitrate after converting nitrate to nitrite. All analyses were performed blinded without knowledge of given treatment.

NGAL in plasma and urine was analyzed using a Pig NGAL ELISA (Kit 044, BioPorto, Gentofte, Denmark). Samples and calibrators were added to a microtiter plate coated with mouse monoclonal antibodies against pig NGAL. Bound NGAL was then detected with another mouse monoclonal antibody labelled with biotin and a streptavidine-HRP conjugate. Samples were analyzed as singletons in batch mode. The total coefficient of variation for the method in this study was estimated at 5%. Urine NGAL levels were corrected for hourly urine output in each animal.

Calprotectin was measured in Lithium-heparin plasma on a Mindray™ BS-380 (Mindray Medical International, Shenzhen, China) with reagents from Gentian (Moss, Norway). The instrument settings for the method were: sample volume = 3 µL, R1 volume = 200 µL and R2 volume = 30 µL. The wavelength was 605 nm. CRP was also analyzed in Li-heparin plasma on an Architect Ci8200 analyzer (Abbott Laboratories, Abbott Park, IL, USA) with reagents from Abbott Laboratories.

6.3 Ethical considerations

Paper I, II and III. These studies were approved by the Animal Ethics Board, Uppsala, Sweden (C215/5 and C225/8, respectively). All animals were handled in strict accordance with the animal experimentation guidelines of the Animal Ethics Board of Uppsala University and the European Convention of Animal Care. Water and food access was ad libitum until 1 h before the experiment.

Paper IV. This observational study was performed in the general ICU of Uppsala University Hospital. The study was approved by the regional ethical review board in Uppsala (No.01/367).
6.4 Statistics

In both paper I and II, calculations of group sizes were based on estimated differences in primary variables of at least 25% with an $\alpha$-error of 0.05, a power of 0.8 and a SD of 10%. Plasma cytokine levels (Papers I and II) were logarithmized and then approximated to normal distribution. Differences between the groups over time were evaluated by analysis of variance for repeated measurements (ANOVA). Student’s t-test was used as outlined in the statistical protocol of each paper. In addition, this test was used in a post-hoc analysis of differences between subgroups in respiratory function (paper I). Statistica (StatSoft, Tulsa, OK) was used for all statistical calculations. T-test was used to assess within-group differences at different time points. All calculations were made in Statistica™ (Statistica version 7.1, Tulsa, OK). A P-value < 0.05 was considered significant. All values were expressed as mean ± SD.

In paper III, data were tested for normality and for baseline differences between the groups. Heart rate, plasma lactate and WBC data were log-transformed to achieve normal distribution. Intergroup differences were assessed with repeated measures ANOVA type III for normally distributed data. If group difference was found, a post-hoc test with unequal N-test for groups ‘EtXNaCl’ vs. ‘HctEtX’, groups ‘EtXNaCl’ vs. ‘EtXHct’ and groups ‘EtXHct’ vs. ‘HctEtX’ was performed. Intergroup differences were assessed for non-normally distributed data with Kruskal-Wallis test. Change over time for non-normally distributed data was assessed with Friedman’s ANOVA. A Spearman Rank Order test was performed to analyze correlation between data. p<0.05 was considered significant. We report values as means with standard deviation (SD) or medians with interquartile ranges (IQRs) as appropriate. Analyses were performed using STATISTICA software, version 13 (StatSoft, Tulsa, OK).

In paper IV, data were tested for normality. When normally distributed, a one-way ANOVA was performed. If a difference between the three groups was found, an unequal N honest significant difference post-hoc test was done to identify the two groups generating the difference. Similarly, for non-normally distributed variables, a Kruskal-Wallis test was performed, followed by a Mann-Whitney test if a difference was found in the first test. Friedman ANOVA was used to assess evolution of non-normally distributed variables. Logistic regression was performed with diagnosis of sepsis and mortality as endpoint and results presented as area under the curve of the receiver-operating characteristic (AUC-ROC). We performed analyses using STATISTICA software, version 13 (StatSoft, Tulsa, OK). p<0.05 was considered significant where relevant.
6.5 Protocols

All pigs that were used in an experiment received continuous endotoxin infusions, (*E. Coli*; 0111:B4; Sigma Chemical Co. St. Louis, MO) obtained from the same batch.

6.5.1 Paper I:

At first, the pigs were randomized to one of three groups: ‘UnExp’ (n = 6), ‘PreExp’ (n = 12), and ‘controls’ (n = 9). Group ‘UnExp’ was merely exposed to a 6 h endotoxemic challenge (starting at ‘Baseline’) at 1 and 4 µg x kg⁻¹ x h⁻¹, respectively (n = 3 in each group). Group ‘PreExp’ was given endotoxin at 0.063 and 0.25 µg x kg⁻¹ x h⁻¹, respectively. Thereafter, they were challenged, at ‘Baseline’ with a 2nd hit of endotoxin for another 6 h, as was group ‘UnExp’, (n = 3 in each group). Animals serving as ‘controls’ (n = 9), were given a 24 h infusion of saline or endotoxin at 1 and 4 µg x kg⁻¹ x h⁻¹, respectively (n = 3 in each group). Controls were only given saline after ‘Baseline’. A schematic picture of this design is given below.
6.5.2 Paper II:

All pigs received a 6 h continuous endotoxin infusion at 2 µg x kg\(^{-1}\) x h\(^{-1}\). The pigs were randomly allocated into two equally-sized groups by the sealed envelope method. When the MPAP increased to twice the baseline value, a single dose of hydrocortisone 5 mg x kg\(^{-1}\) was given intravenously to the pigs in the hydrocortisone group. This steroid dose was chosen because it is in the upper range of what is being used in the intensive care unit at our hospital. The dose used by us is in the same range as previously used both in clinical and experimental settings [116, 117]. The control group received the corresponding volume of saline. After completion of the 6 h endotoxemic period, all pigs were killed by an i.v. overdose of potassium chloride and mechanically terminated according to the local ethical guidelines.

6.5.3 Paper III:

An unbiased caregiver randomly allocated sixteen pigs to one of four equally-sized groups designed as a modified Latin square. The objective of this design was not only to make the most economic use of working hours and research grants, but also to avoid the unnecessary killing of pigs. Three groups received a continuous 6 h endotoxin infusion at 2.0 µg x kg\(^{-1}\) x h (n=12) from baseline. One of these groups received hydrocortisone 30 min prior to baseline, the second group received hydrocortisone when endotoxemic shock was established i.e. MPAP increased to twice the baseline value. The steroid dose chosen was the same as in the previous experiment. The third group received saline in the corresponding volume at endotoxemic shock and served as a control group. The last group (n=4), serving as a non-endotoxemic control group, was given the corresponding volume of saline instead of endotoxin. As the endpoint was a 6 h experimental duration, pigs not surviving the experiment were replaced at a later occasion.
6.5.4 Paper IV:

Plasma calprotectin was analysed in all patients admitted to the ICU in October and in December 2014. This population of patients was screened for discharge diagnoses and allocated to the following groups: septic shock, post-operative care after elective surgery without known infections, and intoxication with no systemic inflammatory response.

Data were extracted from the ICU database where all admissions are registered. In the septic shock group, we aimed to limit other causes of systemic inflammatory response. The following inclusion criteria were therefore set: emergency ICU admittance, at least two SIRS criteria on admission [3], ICD-10 diagnosis codes for septic shock or severe sepsis with organ failure. Patients’ notes and charts were assessed to verify that the patient fulfilled SIRS criteria and had an early onset sepsis.

The patients in the elective post-operative group were included to serve as a group with non-infectious systemic inflammatory response. Patients in this group were therefore identified by having the ICD-10 diagnosis code for uncomplicated post-operative care in the ICU after elective major abdominal or orthopaedic surgery. All patients in this group went through scheduled surgery and were admitted to the ICU directly from the operating theatre.

Finally, in order to include a group of patients as controls without systemic inflammatory response, patients observed in our unit due to intoxication were included. Patients with intoxication were admitted directly from the emergency department.
7. Results

Results from Paper I:

*Differences in Organ Dysfunction in Endotoxin Tolerant Pigs Under Intensive Care Exposed to a Second Hit of Endotoxin.*

A total of thirty animals participated in the experiment. Three animals died during the first two hours of experimental duration. These were randomized to the 6 h endotoxin infusion group, one pig in the group ‘UnExp’ H and the other two in the group ‘UnExp’ L. All three pigs were excluded from the experiment and replaced according to the protocol. There was no difference in any parameters at inclusion time at -24 h and 0 h.

**Interventions**

Fluid: five out of six pigs in the group ‘UnExp’ needed boluses with colloid fluid after baseline (median 25 mL x kg-1, range 20-50 mL), in contrast to the group ‘PreExp’, where one pig received colloid fluid (10 mL x kg-1).

Vasoactive support: five out of six pigs in the group ‘UnExp’ needed support with norepinephrine compared with the group ‘PreExp’, where three animals received this support.

Ventilation: Adjustment of ventilation was indicated and performed according to the protocol in four of the nine groups (in the groups ‘PreExp’ LH and HH, and the groups ‘UnExp’ H and L).

**Outcomes**

TNF-α and IL-6 both responded in a dose-dependent manner to endotoxin tolerance. TNF-α reached its highest level at 1 h in the group that received the high dose of endotoxin at baseline, group H, but this high level diminished and at the end of the experiment, there were no difference between the two groups not pre-exposed to endotoxin. In the pre-treated groups, ‘PreExp’ HH and LH vs. ‘PreExp’ LL and LH, the difference was sustained at both 1 h and at the end of the experiment at 6 h (p<0.05 for both). In the other setting of pre-exposed groups (‘PreExp’ HH and HL vs. ‘PreExp’ LH and LL), there
was a difference at 1 h, $p<0.05$, but not at 6 h. An almost identical pattern of results was seen for IL-6.

WBC decreased in all the animals receiving high-dose endotoxin. This result was less evident in the group of animals receiving high-dose ‘PreExp’ (e.g. HH and LH) compared to the group ‘UnExp’. In the group low-dose ‘PreExp’, the WBC was unchanged or slightly increased in groups LL and LH compared to the low-dose (L) in the group ‘UnExp’, which was decreased ($p<0.05$ at both 2 and 6 h).

LVSWI was better preserved both in the time of the maximal response and in the end of the experiment in the groups ‘PreExp’ as compared to those in the group ‘UnExp’, ($p<0.001$ at 3 h and $p<0.01$ at 6 h).

In contrast, PaO$_2$ / FiO$_2$ in the group ‘PreExp’ did not show any difference at 3 h but at the end of the experiment, the ‘PreExp’ group was more deranged than the ‘UnExp’ group, ($p<0.05$). The low-dose groups, ‘PreExp’ and ‘UnExp’, did not respond with any changes compared to baseline even though there was a difference between the ‘PreExp’ groups and the ‘UnExp’ group at baseline ($p<0.05$).

Static pulmonary compliance responded in a similar way with no difference at the time for maximal response, but at the end of the experiment, the high-

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**Fig. 1. TNF-α levels during the entire experiment in the high-dose endotoxin challenge subgroups.**

Subgroups LH, HH and H received the high-dose endotoxin challenge after baseline, i.e. 4 $\mu$g x kg$^{-1}$ x h$^{-1}$. During the first 24 h, LH and HH received 0.063 and 0.25 $\mu$g endotoxin x kg$^{-1}$ x h$^{-1}$, respectively. SalC is the saline control subgroup. The arrow indicates baseline. The values are given as mean ± SE. * denotes $p<0.05$, Student’s $t$ test comparing subgroups LH and HH.
dose ‘PreExp’ group was more affected than the ‘UnExp’ group, (p<0.05). No difference was detected in the low-dose groups.

Results from Paper II:

Counteraction of early circulatory derangement by administration of low dose steroid treatment at the onset of established endotoxemic shock is not directly mediated by TNF-α and IL-6.

All pigs fulfilled the inclusion criteria. There were no differences at baseline. One pig died during the first hour of the experiment and was replaced according to the protocol. The eight pigs were randomized into two equally-sized groups, ‘EtxHct’ and ‘EtxSaline’.

In all pigs, endotoxemic challenge caused a definite increase in MPAP that was, on average, doubled after 11.3 minutes, (range: 9.5 –17.3 min.). At that time, hydrocortisone 5 mg x kg⁻¹ or the corresponding volume of saline was administered i.v.

The pro-inflammatory cytokines TNF-α and IL-6 increased markedly after the onset of endotoxemia. TNF-α reached its highest value at 1 h, whereas IL-6 spiked at 3 h. Steroid treatment did not significantly blunt these increases.

MAP was better preserved, HR was lower and SVRI was higher in the hydrocortisone plus endotoxin group as compared to the saline plus endotoxin group of animals (p<0.05 for all). In contrast, C.I. did not differ between the groups.

Total nitrite/nitrate decreased approximately 30% over time (0 h vs. 6 h) in both groups, without any significant difference between them.

Fig. 2. Levels of TNF-α and IL-6 during the 6 h experiment. Steroid treatment did not significantly affect the course between the groups ‘EtxHct’ and ‘EtxSaline’.
Both plasma and urine hydrocortisone increased after the hydrocortisone injection. In the group of pigs merely given endotoxin plus saline, increases in urinary hydrocortisone were seen (p<0.05).

In the endotoxin plus saline, but not in the endotoxin plus hydrocortisone group, both body temperature and hemoglobin increased as compared to baseline values, (p<0.05 for both).

Results from Paper III:

*The impact of hydrocortisone treatment on neutrophil granulocyte activation in porcine endotoxemic shock.*

All pigs fulfilled the inclusion criteria. Two pigs died during the experiment. The first pig in group ‘EtxNaCl’ died from circulatory collapse after one hour, the second pig in the group ‘EtxHct’ died at the end of the experiment. Both pigs were replaced at a later occasion as the endpoint of the experiment was to investigate plasma NGAL during six hours of endotoxemia. The four groups did not differ in any values at baseline.

The groups receiving endotoxin infusion and physiological saline or hydrocortisone after established endotoxemic shock responded with a rise in NGAL (p<0.001). Groups receiving hydrocortisone before the endotoxin challenge responded with a diminished level of NGAL in plasma compared with those receiving hydrocortisone when septic shock was established (p<0.05). There was also a negative correlation between plasma NGAL and neutrophil granulocyte counts (R=-0.65) and WBC (R=-0.59). Urine NGAL was lower in non-endotoxemic animals compared to each endotoxemic group (p<0.001 for all), but did not differ between the endotoxemic groups during the experiment.

WBC decreased in each endotoxemic group, but not in the non-endotoxemic controls (‘NaClNaCl’) and was lower in each endotoxemic group when compared to the ‘NaClNaCl’ group (p<0.001) and also when compared to the groups pre-treated with hydrocortisone (‘EtxNaCl’ and ‘EtxHct’ vs. ‘HctEtx’, p<0.01, for both).

HR was higher in all groups receiving endotoxin vs. the non-endotoxemic controls ‘NaClNaCl’, (p<0.001 for all). Endotoxemic pigs pre-treated with hydrocortisone (‘HctEtx’) had lower HR compared to the other endotoxemic groups (‘EtxNaCl’ and ‘EtxHct’, p<0.01 for both). Similarly, MAP was lower in the ‘EtxNaCl’ group than in the other groups, (i.e. groups ‘NaClNaCl’, ‘EtxHct’ and ‘HctEtx’, p<0.001) Fig.3.
Results from Paper IV:

*The performance of plasma calprotectin as a biomarker of early sepsis – a pilot study*

Paper IV is a clinical examination involving 45 of the 118 patients cared for under the observation period in the ICU at University Hospital Uppsala. This pilot investigation consists of patients with sepsis plus two control groups, distributed as septic shock (n=15), post-operative care (n=23), and intoxication (n=7).

Data on laboratory parameters from blood samples taken on admission, vital signs, ventilator and vasoactive treatment and Simplified Acute Physiology Score 3 (SAPS 3) were analyzed. Plasma Calprotectin was measured for up to three ICU days. Day of death was assessed from the national patient register.
SAPS 3 score was higher in the sepsis group compared to post-operative patients, but not in patients in the intoxication group (p<0.05). Core temperature, plasma creatinine, white blood cell count (WBC) and plasma CRP were higher in the sepsis group vs. the two other groups. As almost all patients in the post-operative and intoxication groups were discharged from the ICU the day after admission, repeated plasma calprotectin levels were mostly available in the sepsis group.

Although the highest numerical plasma calprotectin levels were found in the sepsis group, levels on ICU admission were similar in the sepsis and the post-operative groups and both were higher compared with the intoxication group (p<0.01 for both). There was no change in calprotectin values in sepsis patients during the three ICU days.

The performance of calprotectin and CRP in differentiating between intoxication and sepsis, expressed as AUC-ROC, was similarly high (0.95 vs. 0.96). Plasma CRP had a greater AUC-ROC than plasma calprotectin when discriminating sepsis patients from those with post-operative inflammation (0.74 vs. 0.65). Combining plasma calprotectin and CRP increased the AUC-ROC for differentiating sepsis and post-operative patients to greater levels than these biomarkers individually (0.80).

Finally, the performance of plasma calprotectin as a predictor of 30-day mortality was better than any of the other inflammatory variables apart from the composite mortality prediction score SAPS 3 expressed as AUC-ROC (0.70 vs 0.92).
8. Discussion

Plasma levels of TNF-α and IL-6 were reduced by 24 h endotoxin priming before the start of a 6 h endotoxemic challenge, (Paper I). In contrast, plasma levels of TNF-α and IL-6 were not significantly reduced by hydrocortisone given early in the development of endotoxemic shock (Paper II).

Circulatory derangement, i.e. cardiac performance and hypoperfusion expressed by changes in LVSWI and BE, respectively, was less expressed in endotoxin-tolerant pigs. This persisted even when challenged with endotoxin regardless of dose (Paper I). In Paper II, MAP was better preserved in the ‘EtxHct’ group. HR was lower, as were hemoglobin-levels as a sign of diminished vascular permeability. SVRI was higher in the endotoxemic pigs treated with hydrocortisone. This was not explained by an ability of steroids to modulate the production of NO (Nitric oxide), which has been suggested to be a mechanism of steroids in this aspect [116]. NO causes vasodilation and is one of the crucial components implicated in vasoplegia and vascular hypo-reactivity [118]. A low dose of hydrocortisone administered early during the development of endotoxemic shock ameliorates some of the endotoxin-mediated circulatory derangements, e.g. deterioration in MAP and SVRI and increased HR. These effects do not seem to be mediated by TNF-α, IL-6 or NO (Paper II).

The current experiments (Paper III) show attenuated circulatory and acid-base response to endotoxin in pigs treated with hydrocortisone, but this effect was only partly related to the timing of this treatment. This may be contradictory, since the release of NGAL occurs early in the granulocyte differentiation [85] and, given the high turnover and the short half-life of these cells in endotoxemia [119], as well as the short half-life of NGAL [80], one would have expected that the timing of hydrocortisone should not have impacted on neutrophil-activation in these experiments. However, released NGAL has not only bacteriostatic functions, but also an important role in the maturation and activation of neutrophil granulocytes [87, 88], i.e. NGAL-mediated neutrophil activation is a positive feedback loop triggered by, among other factors, endotoxemia.

Nevertheless, pre-exposure to endotoxin did not reduce the pulmonary response to endotoxemic challenge. In fact, both PaO₂/FiO₂ and static pulmonary compliance were reduced in this group when pre-treated with endotoxin at low dose (Paper I). In paper II and III, pulmonary function was not evaluated. The rationale for not having pulmonary function as an endpoint, when
evaluating steroid treatment in endotoxemia, is the fact that previous investigations have not shown any major effect of steroids on respiratory function in such a setting. There is no obvious reason for the lungs to react more to endotoxemic challenge after endotoxemic pre-treatment as compared to endotoxin-naive animals. It is tempting to speculate that this might have a phylogenetic explanation, since the lungs are, more or less, constantly exposed to microbes, which might be regarded as an analogous condition to endotoxin tolerance. Such a mechanism could possibly also be found in man. In a clinical material, ARDS [120] was related to reduced capacity of mononuclear cells to release cytokines TNF-α, IL-6, and IL-1 compared to patients with less expressed lung damage. TNF-α is probably the best marker of endotoxin tolerance, as assessed by its absence following repetitive LPS challenge [121, 122].

Leukocyte count was lower in the endotoxin-naive group compared to the ‘PreExp’ groups at maximal response and at the end of the experiment (p<0.001 and 0.01 respectively, Paper I). When evaluating the ‘PreExp’ groups, leucocytes were found to decrease in all animals in the high-dose challenge groups, while the groups receiving low-dose challenge responded with unchanged or slightly increased levels of WBC. The difference was even more evident compared to the low dose ‘UnExp’ group at 2 h and 6 h, (p<0.05, for both, Paper I). In clinical reality, septic shock occurring secondary to trauma, major surgery or an initial infection may therefore result in a diminished inflammatory response, which must be taken into consideration.

In Paper III, hydrocortisone was administered prior to the endotoxin infusion and at the onset of endotoxemic shock, but not subsequently. The inflammatory response, including NGAL and white blood cell counts, was diminished and a negative correlation between plasma NGAL and neutrophil granulocyte counts (R=-0.65) and WBC (R=-0.59) was seen. We have interpreted this as a negative feed-back loop due to diminished levels of neutrophils caused by low levels of NGAL. We therefore postulate that decreased NGAL levels may inhibit the additional recruitment of neutrophils, thereby limiting the inflammatory response. The importance of NGAL in the neutrophil-mediated inflammatory response has been described in NGAL-deficient mice and men [87].

The inflammatory response in the body reacts very quickly when challenged with an infusion of endotoxin and one of the promptest reactions is the release of NGAL from neutrophil granulae via the TLR4 (toll-like receptor 4) [93]. Plasma NGAL increases during systemic inflammatory response triggered by endotoxin infusion. This inflammatory response is attenuated for several hours by hydrocortisone administration prior to the start of endotoxin infusion, suggesting that the effect on plasma NGAL levels is a very early phenomenon in endotoxemia (Paper III).
To our knowledge, the diminished increase in plasma NGAL with hydrocortisone pre-treatment in the current study has not been reported previously in endotoxemia or sepsis. However, these findings correspond to those noted in other conditions such as AKI, where reduced oxidative stress [123] and attenuated neutrophil granulocyte response were suggested as possible mechanisms of steroid pre-treatment on NGAL levels [124]. Steroids could potentially inhibit the production on NGAL through the IKKB/Nf-KB pathway that is suppressed by these drugs [125]. The debate concerning steroids and sepsis has been going on for decades. Although we administer hydrocortisone at an early time point in the development of endotoxic shock, timing of steroid administration is probably most important for this effect on the systemic circulation. Park et al. found that steroids administered to patients in septic shock with less than 6 h of hypotension were significantly associated with better outcome than steroids administered after 6 h of hypotension caused by septic shock [22].

Somewhat surprisingly, we noted that the hydrocortisone levels in urine increased significantly, also in the control animals that were not steroid-treated (Paper II). These changes were interpreted as a response to the stress that septic shock brings to the organism. Whether these increases are accompanied by pre-receptor up-regulations of cortisol, altered receptor density or gene transcription changes cannot be determined from the present study, but they may indicate that increased steroid activity belongs to the normal response to a septic stimulus [126].

Urine NGAL was higher in each endotoxemic group compared to the non-endotoxemic group, but hydrocortisone treatment did not affect urine NGAL levels. NGAL levels correlated (inversely) to neutrophil granulocyte count but not urine output, suggesting that the NGAL response to endotoxemia was primarily related to the extent of systemic inflammation, not to kidney injury, (Paper III).

One clinical implication of this study (Paper III) is to underline the value of early, or pre-emptive, low-dose steroid treatment to limit SIRS. Another clinical implication is to point out the possibility that patients having steroid treatment initiated before the onset of a severe infection leading to a septic condition may exhibit less obvious clinical signs of sepsis, thus obscuring this diagnosis. Hydrocortisone administration attenuates the effects of endotoxemia on the circulation, but low NGAL levels may also lead to diminished microbial defense, a phenomenon described in clinical studies in septic shock patients [18]. Monitoring plasma NGAL could be a method to follow steroid-induced immunosuppression.
Patients with sepsis and patients admitted for post-operative care after major surgery had higher plasma calprotectin on ICU admission than patients admitted due to intoxication, suggesting that plasma calprotectin levels are markedly increased in systemic inflammatory response (Paper IV). Although numerically higher in the sepsis-group, plasma calprotectin levels did not differ between patients with sepsis and those admitted for post-operative care. Plasma calprotectin levels did not change in patients with sepsis during the first 3 days after ICU admission.

Calprotectin is a calcium-binding protein constituting the integral part of both cells involved in inflammatory processes, such as neutrophil granulocytes and monocytes, and also in epithelial cells [127]. Increased levels of calprotectin indicate the activation of these cells [128, 129] as occurring on activation of the innate immune system [130]. This protein is probably not just a byproduct of neutrophil granulocyte lysis, but also one with endogenous bacteriostatic effects [96]. The traditional habitat of calprotectin has been the diagnostics of inflammatory bowel disease through measuring this biomarker in faeces [131]. However, calprotectin has been investigated for many conditions where activation of leukocytes is suspected [132-135].

Plasma CRP was better at discriminating sepsis from post-operative inflammation compared with plasma calprotectin, while both biomarkers were better together than separately at accurately discriminating presence versus absence of systemic inflammation. In our opinion, plasma calprotectin increases promptly and remains constant. CRP, on the other hand, peaks after approximately 20 h. Regardless of the composite mortality prediction score, SAPS 3, plasma calprotectin performed better than any of the other inflammatory variables in predicting mortality at 30 days.

Although plasma calprotectin has been suggested as a biomarker of sepsis over the past decades [136, 137], systematic evaluation of this protein has only begun in the recent years. At least two studies assessing the usefulness of serum calprotectin in diagnosis of sepsis in neonates with positive blood cultures compared to neonates with suspected sepsis without positive cultures reported good diagnostic accuracy [104, 138]. However, only one study investigated the role of plasma calprotectin to aid the diagnosis of sepsis in adult ICU patients [139]. Similar to our study, the authors Gao S et al report excellent performance of plasma calprotectin in discriminating patients with sepsis from controls without SIRS. However, when comparing to patients with SIRS, a considerable overlap is seen in calprotectin levels between patients with and without infections. One difference between the study by Gao S et al and our study is that blood samples in the latter study, used in the main analysis, were taken on ICU admission when the diagnostic uncertainty is commonly the greatest. Another difference is that our study reports the use of a turbidometric method that enables automated routine calprotectin analysis and results within 30 minutes, i.e. an analysis method that is well suited for emergency care.
A recent study using the same analysis method as our study reported an upper cut-off of 2.6 mg x L$^{-1}$ as reference value for plasma calprotectin, a cut-off corresponding well to our data [140]. 75% of the patients with sepsis in the current study had a calprotectin level above this limit, while none of the non-inflammatory controls observed in the ICU after intoxication had calprotectin levels above the suggested cut-off for the reference interval in the general population.

This study (Paper IV) suggests that calprotectin is one of the candidate biomarkers for the early diagnosis of sepsis and SIRS. Based on the results of this pilot study in the early phase of sepsis and SIRS, plasma calprotectin in the reference range suggests that sepsis is unlikely.

### 8.1 Strengths and limitations

This is, as far as we know (Paper III), the first report on the effect and timing of hydrocortisone administration on NGAL levels in plasma and on linking this to neutrophil granulocyte counts in endotoxemia. Other strengths include that our findings on the effect of timing of hydrocortisone replicate previous findings. The results are further supported by the design with four groups, including control groups, for the effects of endotoxin and of hydrocortisone.

However, studies (Paper I, II, and III) with research animals have a number of limitations. The studies were conducted in an endotoxemic model on young and previously healthy animals, which limits its validity for patients seen in intensive care. Nevertheless, endotoxemia induces the innate immune system, as does sepsis, and investigating reactions of endotoxin priming or the timing of steroid administration are unethical and difficult to realize in the ICU.

Secondly, although many animals were included in total in the studies, there were only a few animals in each group. Although this limits the power of the study, the positive results are discussed. Thus, the effect of beta error is limited.

Finally, a clear limitation of the animal studies is the short observation period.

To our knowledge, this (Paper IV) is the first study describing the performance of plasma calprotectin as a marker of sepsis and a mortality-predictor in an adult ICU population, as well as its feasibility for rapid turnaround analysis. In order to decrease selection bias, all adult patients admitted to the ICU during the study period were screened for sepsis, post-operative care or intoxication as discharge diagnosis. Moreover, in patients that were identified with these diagnoses, the accuracy of the diagnosis as well as presence of predefined illness criteria (e.g. fulfilment of SIRS criteria on admission) were verified. Finally, the analytic method for plasma calprotectin investigated in this
study is possible to set up as a relatively cheap acute routine analysis. Thus, the results of this study can aid further evaluation of this biomarker in an emergency environment.

On the other hand, this study (Paper IV) has several limitations. The number of patients is low in this pilot investigation, clearly limiting the conclusions that can be drawn. However, these data suggest that measuring plasma calprotectin in this population is feasible and the results of this study can aid designing future studies.

As in all sepsis biomarker studies, a limitation is the relatively broad definition of sepsis syndrome with variable presentation. In an attempt to include a somewhat uniform group of patients with sepsis, only patients admitted with new onset sepsis were included in the study, thus limiting the generalization of our findings. Another challenge with sepsis biomarker studies is the lack of a generally accepted biomarker golden standard for sepsis. We decided to describe the performance of plasma calprotectin parallel with CRP as it is the standard sepsis biomarker locally. However, comparisons to other biomarkers such as procalcitonin and heparin binding protein were not made in our study. Additionally, the traditional way of testing a biomarker is to quantify the biomarker in individuals confirmed to have the condition investigated and in those whom the condition is suspected but unconfirmed. This approach could not be applied in the current study as the diagnosis of sepsis is clinical and based on criteria that have relatively low specificity in the ICU population [141]. Moreover, confirmation of the diagnosis is difficult due to the relatively low number of positive cultures [142]. A common clinical scenario is, however, where a decision on the administration of antibiotics has to be made in patients with critical illness at the latest on ICU admission. We therefore designed the study where patients admitted to the ICU with sepsis were compared with patients admitted after major surgery resulting in a non-infective systemic inflammatory response, as well as with a third group of patients with intoxications i.e. without inflammatory response. Finally, the performance of plasma calprotectin as a marker of mortality was higher than for many other individual biomarkers. However, these findings should be interpreted in the context that all deaths occurred in the sepsis group, suggesting that the conclusions regarding mortality risk can only be applied to this group of patients.
9. Future plans

Studies with longer observation periods as well as studies in humans would increase our understanding of how the timing of steroid treatment influences the anti-inflammatory effects of steroids. Monitoring plasma NGAL could be a method to follow steroid induced immunosuppression.

Future studies should focus on investigating the usefulness of plasma calprotectin as a sepsis biomarker in larger patient cohorts, preferably in a multicentre study. Also, broadening the patient populations investigated to postoperative sepsis, viral and fungal sepsis and sepsis in immunocompromised patients would be of interest. Finally investigating the response rate of plasma calprotectin after infectious insult and its change over the course of sepsis would add to the cumulative knowledge on this biomarker.
10. Conclusions

When animals pre-exposed to endotoxin were challenged with a 4 to 64-fold increase in endotoxin dose after baseline, there was a significant attenuation in the TNF-α response compared to animals not pre-exposed to endotoxin. In this TNF-α response, an obvious dose dependency in endotoxin tolerance could be demonstrated.

Endotoxemic animals treated with hydrocortisone were more stable in circulatory variables than those without such treatment (Paper II and III). These endotoxin-mediated derangements do not seem to be mediated by TNF-α, IL-6 or NO. Pre-treatment with hydrocortisone attenuated the neutrophil granulocyte response and consequently diminished the release of NGAL in plasma. Circulatory derangement was associated with high plasma NGAL levels.

Plasma calprotectin levels on ICU admission are a sensitive marker of systemic inflammation and are more markedly increased in patients with sepsis and in patients with SIRS, than in patients admitted due to intoxication, i.e. without SIRS.

Plasma calprotectin performed better than any of the other inflammatory variables in predicting mortality at 30 days, except for the composite mortality prediction score, SAPS 3.
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