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DIAGNOSTIC AND PROGNOSTIC MARKERS IN SEPSIS

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**Karolinska
Institutet**

Stockholm 2013

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ISBN 978-91-7549-288-9

*“If a man does not keep pace with his companions,
perhaps it is because he hears a different drummer.
Let him step to the music which he hears,
however measured or far away.”*

-HENRY DAVID THOREAU



**Karolinska
Institutet**

Institutionen för medicin, Huddinge

Diagnostic and prognostic markers in sepsis

AKADEMISK AVHANDLING

som för avläggande av medicine doktorsexamen vid
Karolinska Institutet offentligen försvaras i Welandersalen,
ingång B2, plan 00, Karolinska Universitetssjukhuset, Solna

Fredagen den 22 november 2013, kl. 09:00

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ABSTRACT

Sepsis is a life-threatening disease affecting millions of people globally. The more severe forms are considered to be a consequence of an unbalanced systemic inflammatory response to infection, causing organ dysfunction, vascular leakage and hypotension. An early diagnosis followed by appropriate antimicrobial therapy is critical for the outcome. Conversely, inappropriate antibiotic use will escalate antibiotic resistance. Therapeutic guidance from microbiological cultures is lacking in the early hospital course, and better tools are needed for prompt identification and severity stratification of sepsis patients.

The aim of this thesis was to assess the clinical impact of severe sepsis and the diagnostic properties of clinical and biological markers in patients with a suspected or established serious infection.

The diagnostic value of clinical and laboratory variables in predicting infections that require antibiotic treatment was evaluated in a prospective observational study of adult patients with suspected severe infections. We also analyzed the relations between severe sepsis, systemic inflammatory response syndrome (SIRS), and the clinical course.

We concluded that increased C-reactive protein, white blood cell count, respiratory rate and a decreased hemoglobin level contributed independently to an accurate selection of patients for antibiotic therapy. Procalcitonin did not provide guidance on antibiotic decisions, but was associated with bacteremia and severe sepsis. In addition, severe sepsis was a common condition (42%), but mortality was low (5%), suggesting that severe sepsis is a more benign condition than earlier reported. SIRS did not exhibit discriminative ability in the classification of sepsis.

We used the enzyme-linked immunospot (ELISpot) assay to study the spontaneous as well as the lipopolysaccharide (LPS)-induced secretion of a number of pro- and anti-inflammatory cytokines from leukocytes of septic patients and healthy controls. We concluded that circulating leukocytes did not appear to be the source of the increased plasma levels of cytokines observed in sepsis. A selective sepsis-induced downregulation of cytokine secretion in response to LPS was found: while the numbers of IL-6 and TNF- α secreting cells remained similar, significantly fewer IL-1 β , IL-10, IL-12p40 and GM-CSF secreting cells were seen in samples from septic patients as compared to healthy controls. The reduced number of cytokine secreting cells in response to LPS stimulation correlated with disease severity.

LPS-induced cytokine secretion from polymorphonuclear cells (PMN) and peripheral blood mononuclear cells (PBMC) from healthy donors was analyzed by ELISpot. PMN were found to secrete the two chemokines IL-8 and MIP-1 β in response to LPS. Also TNF was secreted but by significantly fewer cells. PBMC had a broader cytokine secreting repertoire and released considerably larger amounts of the investigated cytokines, with CD14⁺ monocytes being the primary source of production.

LIST OF PUBLICATIONS

- I. **Clinical and laboratory variables identifying bacterial infection and bacteraemia in the emergency department**
Gille-Johnson P, Hansson KE, Gårdlund B.
Scandinavian Journal of Infectious Diseases 2012;44(10):745-52.

- II. **Severe sepsis and systemic inflammatory response syndrome in emergency department patients with suspected severe infection**
Gille-Johnson P, Hansson KE, Gårdlund B.
Scandinavian Journal of Infectious Diseases 2013;45(3):186-93.

- III. **Circulating monocytes are not the major source of plasma cytokines in patients with sepsis**
Gille-Johnson P*, Smedman C*, Gudmundsdotter L, Somell A, Nihlmark K, Paulie S, Andersson J, Gårdlund B.
Shock 2012;38(6):577-83.

- IV. **ELISpot analysis of LPS-stimulated leukocytes: human granulocytes selectively secrete IL-8, MIP-1beta and TNF-alpha**
Smedman C, Gårdlund B, Nihlmark K, Gille-Johnson P, Andersson J, Paulie S.
Journal of Immunological Methods 2009;346(1-2):1-8.

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LIST OF ABBREVIATIONS

ACCP	American College of Chest Physicians
APC	antigen-presenting cells
AUC	area under the ROC curve
CARS	compensatory anti-inflammatory response syndrome
CRP	C-reactive protein
DAMP	danger associated molecular pattern
DIC	disseminated intravascular coagulation
ED	emergency department
EGDT	early goal-directed therapy
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunospot assay
GM-CSF	granulocyte-macrophage colony-stimulating factor
Hb	hemoglobin
HBP	heparin-binding protein
HLA	human leukocyte antigen
ICU	intensive care unit
IFN	interferon
IL	interleukin
IL-1ra	IL-1 receptor antagonist
INR	prothrombin time
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide (=endotoxin)
MAP	mean arterial blood pressure
MIF	macrophage migration inhibitory factor
MIP-1 β	macrophage inflammatory protein 1 β
NF- κ B	nuclear factor κ B
NGAL	neutrophil gelatinase-associated lipocalin
NO	nitric oxide
PAF	platelet activating factor
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCT	procalcitonin
PMN	polymorphonuclear cells (=granulocytes)
PRR	pattern-recognition receptor
ROC	receiver operating characteristic
RR	respiratory rate
SCCM	Society of Critical Care Medicine
SIRS	systemic inflammatory response syndrome
sTREM-1	soluble triggering receptor expressed on myeloid cells-1
suPAR	soluble urokinase-type plasminogen activator receptor
TGF- β	transforming growth factor β

TLR	toll-like receptor
TNF	tumor necrosis factor
WBC	white blood cell count

1 BACKGROUND

1.1 THE PROBLEM

1.1.1 A persistently deadly disease

Case 1, ancient Greece:

“Criton, in Thasus, while still on foot, and going about, was seized with a violent pain in the great toe; he took to bed the same day, had rigors and nausea, recovered his heat slightly, at night was delirious. On the second day, swelling of the whole foot, and about the ankle erythema, with distension, and small bullae; acute fever; he became furiously deranged; alvine discharges bilious, unmixed, and rather frequent. He died on the second day from the commencement.” [1]

Case 2, Stockholm, Sweden, 21st century:

A previously healthy male, age 61, presented to the emergency department at the hospital with an infected wound acquired when bathing. He was given antibiotic treatment against "erysipelas". While at work 2 days later onset of nausea, blurred vision, rigors and a swollen, aching lower leg. Physical/laboratory examination: Hypotension, altered mental status, coagulation abnormalities. Thickened and discoloured lower limb with blisters. Progression to multiple organ failure. Interventions: broad antibiotic therapy; repeated surgery; hyperbaric oxygen treatment; intensive organ support, including renal replacement therapy. On the third day of hospital stay, he died.

Although some 2400 years of accumulated medical knowledge separate these two cases of sepsis, they exhibit striking similarities in that they share not only the clinical picture, but also the fatal outcome. They illustrate the fact that modern day clinicians, despite clinical skills and tools immeasurably superior to those available to Hippocrates and his peers, still have to tolerate being reduced to helpless bystanders when a microbial assault strikes with full force.

1.1.2 The magnitude

The true impact of sepsis on modern society is not known. There are no population-based prospective cohort studies, and most estimates are based on studies of hospital discharge data. A diversity of defining criteria has been used in these investigations, resulting in substantial variability in calculations of incidence and mortality between studies [2]. One frequently cited U.S. study found the incidence of severe sepsis to be 300 per 100,000 people, of which half required intensive care and 28.6 % died [3]. The incidence and total mortality seem to be increasing [2, 4-6], likely reflecting ageing and more vulnerable populations. Crude estimates of fatality rates vary between 20-50% for severe sepsis and 40-80% for septic shock. Recently there has been an encouraging trend toward decreasing case fatality rates, possibly due to earlier recognition and treatment [2, 7]. However measured, sepsis remains a major health problem, affecting millions of people globally, and with persistently high mortality rates [3, 8-10].

The most common cause is pneumonia, accounting for slightly less than half of the cases, followed by abdominal and urinary tract infections [8, 11, 12].

Blood cultures are generally positive in about a third of cases, and another third remain of indeterminate microbiological origin [8, 12, 13]. The most commonly found causative organisms are *E. coli*, *Staphylococcus aureus* and pneumococci [12-14].

1.2 THE WORD

The word “sepsis” (σηψις) is of Greek origin and refers to the decomposition of organic matter. It was used by Hippocrates to describe harmful, putrid infection [15, 16]. After antiquity, the term was used occasionally until the mid 19th century. During this period great progress within the fields of anatomy and pathology had been made, which, together with the recent introduction of anaesthesia in 1846, paved the way for a wider use of surgery. Operative procedures were, however, heavily burdened by severe infectious complications, often with fatal results [17, 18]. “Sepsis”, together with the synonymous “septicaemia”, was now increasingly used to describe the general infection, “blood poisoning”, which often accompanied an inflamed wound. The terms became established and were introduced into medical textbooks and dictionaries [19, 20].

1.3 THE DISEASE

1.3.1 Clinical picture, diagnosis and definitions

Although the signs and symptoms of sepsis may be highly variable, it takes only little experience of acute care in order to recognize the usual clinical picture, characterized by fever, chills, general malaise and signs of physiological strain. Still, defining specific and useful diagnostic criteria for sepsis has remained a complicated issue ever since the modern usage of the term was introduced. The predicament is exemplified in a text from that epoch: “During the years 1870 to 1872 alone, more than forty original researches on sepsis...were published...And yet at the present time... it is not even possible to give a general definition of the term..., which could correctly represent all the different conceptions of its nature” [21]. The frustration contained in these words was to be inherited by successive generations of the medical community, despite an enhanced knowledge of underlying pathophysiological mechanisms. The lack of uniform definitions led to great disparities between study populations, and sepsis trials were marred by poor generalizability [22]. In an attempt to resolve this problem, a consensus conference of the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) introduced new diagnostic definitions in the early 1990s, with the intention to provide criteria that were apt for both clinical practice and standardization of research protocols [23]. Central to the proposed definitions was the presence of a “systemic inflammatory response syndrome” (SIRS), a physiologic reaction commonly seen in intensive care patients in response to conditions like trauma, burns and infection. SIRS was defined by at least two of the following signs: an abnormal body temperature, tachycardia, tachypnea or an abnormal white blood cell count (WBC) (Box 1). Sepsis was defined as SIRS caused by an infection (Fig. 1). It was also suggested that SIRS could progress into a more severe state of organ failure, coined “multiple organ dysfunction syndrome” (MODS).

To avoid confusion, the terms “septicaemia” and “septic syndrome” were abolished, while “severe sepsis” was defined as sepsis associated with organ dysfunction, hypoperfusion, or hypotension. Finally, the term “septic shock” was reserved for cases of severe sepsis with hypotension refractory to fluid replacement. “Sepsis”, “severe sepsis” and “septic shock” now defined three progressive disease stages of increasing severity (Fig. 2).

BOX 1. SIRS-defining criteria. Data adapted from Bone et al. [23]

Two or more of the following:
 Temperature >38°C or <36°C
 Heart rate >90/min
 Respiratory rate >20/min or PaCO₂ <4,3 kPa
 White blood cell count >12x10⁹/l or <4x10⁹/l, or >10% immature forms

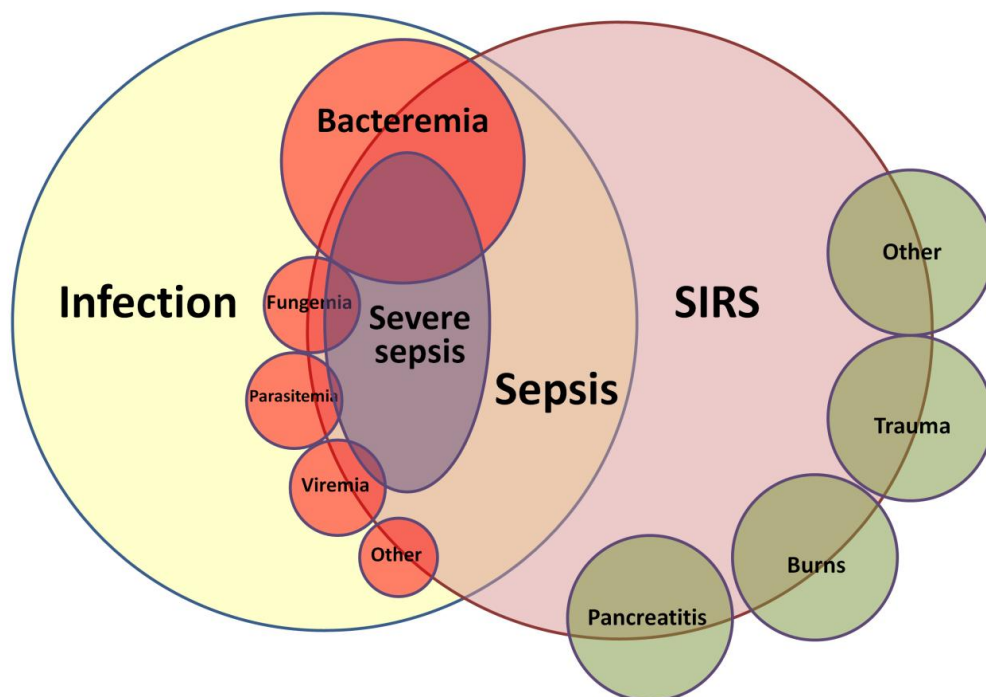


FIGURE 1. Interrelationship between infection, systemic inflammatory response syndrome (SIRS), sepsis, and associated conditions. Adapted from Bone et al. [23]

1.3.1.1 Dear SIRS, were you aware of what you did?

The novel definitions facilitated comparisons between studies, and consequently became a standard for the enrolment of patients in sepsis trials [24]. Criticism was,

nevertheless, soon to follow. SIRS criteria were considered overly inclusive as they were readily met by large numbers of patients across the spectrum of acute care, regardless of disease severity [25, 26]. On the other hand, the definition could also be too restrictive when otherwise overtly septic patients did not satisfy the criteria, and were prevented from study enrolment [27, 28]. As a result, the original ACCP/SCCM criteria were revisited by a second consensus conference in 2001 [29]. The concepts of sepsis, severe sepsis, and septic shock were acknowledged, but in order to facilitate a bedside diagnosis of sepsis, the original SIRS criteria were expanded into a smorgasbord of parameters, ready to pick from by the clinician (Box 2). Although these criteria provided a more complete description of sepsis as a clinical entity, it also made the diagnosis more diffuse and subject to interpretation. The fact that hypoperfusion, hypotension and organ dysfunction parameters, which define severe sepsis, were now introduced among the sepsis (only) defining parameters, only aggravated this situation. Hence, researchers continue to use the original 1991 sepsis criteria and SIRS definition (Box 1) for the enrolment of patients in sepsis trials [12, 30-32].

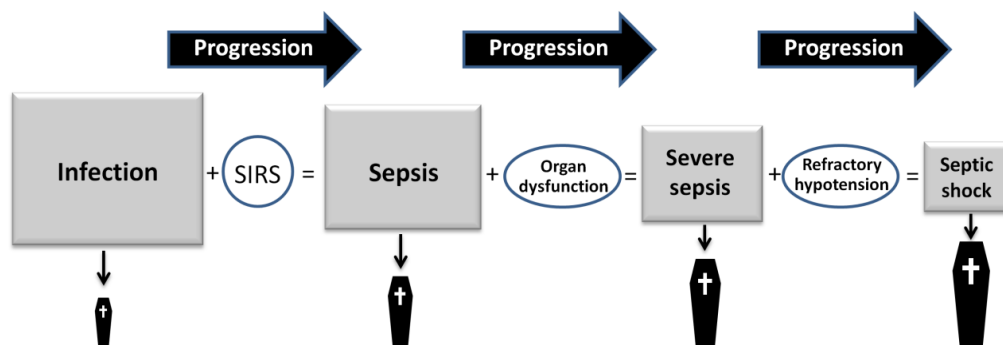


FIGURE 2. The progressive severity stages of sepsis. Increasing severity is characterized by lower incidence and increased case fatality rates.

1.3.2 Current understanding of sepsis pathogenesis

The last decades have seen extraordinary advances in the understanding of the molecular mechanisms that regulate the host response to infection. The following paragraphs attempt to briefly summarize some of the key findings in relation to sepsis.

1.3.2.1 Proinflammatory mechanisms

1.3.2.1.1 Pattern-recognition receptor activation

When pathogens invade otherwise sterile tissues, immune cells initiate a host defense response triggered by the recognition of conserved pathogen associated molecular patterns (PAMPs) through a number of receptors known as pattern-recognition receptors (PRRs). Moreover, PRRs can react to tissue damage through the recognition of endogenous mediators released from dying cells. These mediators are commonly referred to as danger associated molecular patterns (DAMPs), and may explain the SIRS elicited by non-infectious injury like trauma or burns. In sepsis both PAMPs and DAMPs contribute to the inflammatory process.

<p>Sepsis: documented or suspected infection <i>and</i> some of the following:</p> <p>General parameters</p> <ul style="list-style-type: none">Fever (core temperature $>38.3^{\circ}\text{C}$)Hypothermia (core temperature $<36^{\circ}\text{C}$)Heart rate >90 bpm or >2 SD above the normal value for ageTachypneaAltered mental statusSignificant edema or positive fluid balance (>20 ml/kg over 24 h)Hyperglycemia (plasma glucose >120 mg/dl or 7.7 mmol/l) in the absence of diabetes <p>Inflammatory parameters</p> <ul style="list-style-type: none">Leukocytosis (white blood cell count $>12 \times 10^9$/l)Leukopenia (white blood cell count $<4 \times 10^9$/l)Normal white blood cell count with $>10\%$ immature formsPlasma C reactive protein >2 SD above the normal valuePlasma procalcitonin >2 SD above the normal value <p>Hemodynamic parameters</p> <ul style="list-style-type: none">Arterial hypotension (systolic blood pressure <90 mmHg, mean arterial pressure <70, or a systolic blood pressure decrease >40 mmHg in adults or <2 SD below normal for age)Mixed venous oxygen saturation $>70\%$Cardiac index >3.5 l/min/m² <p>Organ dysfunction parameters</p> <ul style="list-style-type: none">Arterial hypoxemia ($\text{PaO}_2/\text{FIO}_2 <300$ mmHg or <40 kPa)Acute oliguria (urine output <0.5 ml/kg/h for at least 2 h)Creatinine increase ≥ 0.5 mg/dl or ≥ 45 $\mu\text{mol/l}$Coagulation abnormalities (international normalized ratio >1.5 or activated partial thromboplastin time >60 s)Ileus (absent bowel sounds)Thrombocytopenia (platelet count $<100,000/\mu\text{l}$)Hyperbilirubinemia (plasma total bilirubin >4 mg/dl or 70 mmol/l) <p>Tissue perfusion parameters</p> <ul style="list-style-type: none">Hyperlactatemia (>3 mmol/l)Decreased capillary refill or mottling <p>Severe sepsis: sepsis with organ dysfunction, hypoperfusion or hypotension</p> <p>Septic shock: severe sepsis with hypotension refractory to intravenous fluid replacement</p>

Toll-like receptors (TLRs), a group of transmembrane proteins, belong to the PRR family and are central to the initiation of a cellular immune response. This intricate process can be exemplified by the TLR4 recognition of bacterial lipopolysaccharide (LPS, also known as endotoxin), a most potent PAMP: Circulating LPS-binding protein (LBP) forms a complex with LPS, which binds to the receptor CD14 on monocytes/macrophages. TLR4 binds to the LBP-LPS-CD14 complex via an auxiliary protein, MD2, and subsequently activates an intracellular signal-transduction pathway that releases transcription factors like nuclear factor κB (NF- κB). Activated NF- κB

moves from the cytoplasm to the nucleus and induces transcription of genes coding for a multitude of proinflammatory proteins, e.g. the cytokines tumor necrosis factor (TNF), interleukin-1 (IL-1) and IL-6. TNF and IL-1 are early and potent initiators of the inflammatory response cascade, sharing an individual capacity to inflict sepsis-like organ failure and circulatory collapse when given in high doses. In contrast, IL-6 does not cause tissue damage or shock, but is a principal inducer of fever and the acute phase response. This involves a shift in the production of hepatic plasma proteins toward a profile more adapted to host defence, with increased release of numerous proteins, including complement factors, LBP and C-reactive protein (CRP), which is also a clinically important inflammatory marker [33-36].

The resulting acute inflammation has potent effects on the local microcirculation, characterized by vasodilation, increased vascular permeability, and passage of leukocytes and circulating immune mediators into the site of infection.

1.3.2.1.2 Leukocytes

Leukocytes, blood cells of the immune system, can roughly be divided into polymorphonuclear cells (PMN), also called granulocytes, and mononuclear cells (PBMC).

Neutrophils are PMN that form the majority of the blood's leukocytes. They play a key role in the frontline host defense to bacterial infection, and an accumulation of neutrophil granulocytes is considered the hallmark of acute inflammation. The cytoplasm is filled with granules containing numerous anti-microbial substances, which will be released (degranulate) in response to inflammatory signals. Neutrophils are rapidly recruited to injured or infected tissues, attracted by inflammatory mediators like TNF, IL-1, IL-8 and nitric oxide (NO), expressed by activated tissue macrophages and endothelium. They roll along and adhere to the vessel wall, before they transmigrate into the tissue where they phagocytose, damage and kill microorganisms. Granule content is released into tissues in order to kill extracellular microorganisms, but can also cause collateral tissue damage. Neutrophils secrete only moderate amounts of cytokines, predominantly with a chemotactic profile, e.g. IL-8, which is central for the recruitment of additional neutrophils. After phagocytosis, they undergo apoptosis and are cleared from the inflammatory site by macrophages in an anti-inflammatory manner [33, 37].

Monocytes are mononuclear cells that constitute 5-10% of circulating leukocytes. Most monocytes are predestined to migrate into tissues where they mature into resident macrophages, specialized to protect their microenvironment and to repair tissue. Like neutrophils, monocytes/macrophages kill microbes through phagocytosis, but they also orchestrate the immune response via a plethora of mediators. This includes prostaglandins, leukotriens, platelet activating factor (PAF) and a wide array of cytokines. In addition, monocytes/macrophages are also antigen-presenting cells (APCs), carrying human leukocyte antigen (HLA) receptors with the capability to ligate and display foreign antigens to antigen-specific T-lymphocytes, thus constituting an important link between the innate and specific immune defense [33, 38].

Lymphocytes make up the larger part of PBMC and represent the specific immune defense. They circulate between the blood and lymphoid organs and are activated by specific microbial antigens presented by APC. Lymphocytes attack the antigen, either by antibodies produced by B-lymphocytes, cytotoxic granules from cytotoxic

T-lymphocytes, or by the recruitment of macrophages and other immune cells by helper T-lymphocytes. A fully activated specific immune response requires considerably more time than the immediate innate response, often weeks [33].

1.3.2.1.3 The coagulation system and the endothelium

The coagulation system is triggered by expression of tissue factor on IL-6 activated endothelium and monocytes, resulting in thrombin generation and enhanced platelet adhesion and aggregation. Together with impaired anticoagulant mechanisms, a procoagulant state with local fibrin deposition is elicited, contributing to the containment of the infection, but also tissue hypoperfusion.

While an increased endothelial permeability is a prerequisite for the extravasation of neutrophils and plasma into the site of infection, an exuberant inflammatory response may lead to apoptosis and detachment of endothelial cells, with an ensuing loss of barrier function [33, 39, 40].

1.3.2.1.4 The complement system

In parallel with phagocytosis, the complement system forms the principal antibacterial system of the immune defense. Subsequent to immune activation, this system of more than 30 plasma proteins and receptors contributes to the elimination of microbes by two key mechanisms: opsonization and cell lysis. Opsonization facilitates phagocytosis by the adhesion of complement proteins to the bacterial cell surface, while cell lysis is caused by a membrane attack complex consisting of complement factors C5-9. This process is initiated by the cleavage of C5 into C5a and C5b. Together with C6-9, C5b form the membrane attack complex, while C5a has anaphylatoxin activity, increases vascular permeability and recruits inflammatory cells to the site of infection. Elevated levels of several complement factors have been associated with sepsis, and a number of studies have pointed to C5a as a significant inducer of hemodynamic and coagulatory perturbation [33, 35, 41].

1.3.2.1.5 The peril

Although essential to the immune response, it is believed that high levels of PAMPs from invading microorganisms can, in concert with DAMPs from damaged host tissue, lead to an overstimulation of immune cells which leads to the uncontrolled systemic response seen in fulminant cases of sepsis. The exact mechanisms by which sepsis causes multiple organ failure remain unclear, but the general conception is that macro- and microvascular effects due to widespread hyperinflammation and coagulation compromise tissue perfusion and oxygen delivery. Vasodilation, increased vascular permeability and depressed myocardial function lead to a pathophysiologic phenotype distinguished by hypovolemia and hypotension, while a hypercoagulant state results in microvascular thrombosis, in its most extreme form termed disseminated intravascular coagulation (DIC). Sepsis has also been demonstrated to impair mitochondrial function, possibly contributing to organ dysfunction [34, 42].

1.3.2.2 *Anti-inflammatory mechanisms and immunosuppression*

In the cross-talk between the systems of pattern-recognition, complement and coagulation there is an abundance of counter-regulatory mediators aiming to modulate

an excessive proinflammatory response. Among these are anti-inflammatory cytokines like IL-10, IL-1 receptor antagonist (IL-1ra), and transforming growth factor β (TGF- β). IL-6, in contrast to its proinflammatory properties, also exhibits anti-inflammatory effects, illustrating the complexity of cytokine interactions. IL-6 inhibits the production of IL-1 and TNF and stimulates the release of IL-10 and cortisol [33]. Circulating immune cells develop diminished antigen presenting capacity and responsiveness to secondary inflammatory stimulation, which in combination with negative regulators of TLR signaling and inducers of immune cell apoptosis contribute to an anti-inflammatory pattern. In addition, there is a neuro-endocrine-immune network in which the cholinergic nervous system responds to efferent signals by the release of acetylcholine, which inhibits proinflammatory cytokine release through ligation to acetylcholine receptors on macrophages (Fig. 3) [35, 36].

There is mounting evidence that critically ill patients who survive the initial phase of sepsis are subjected to a more immunosuppressive condition, with a propensity to develop secondary opportunistic infection and reactivation of latent viral infection [43, 44].

1.3.3 The aggressors

When reviewing the literature on sepsis definitions, it is striking how little interest that is devoted to the very roots of the disease - the microorganisms. A basic understanding of the microbial perspective is, however, fundamental to the understanding of sepsis.

With some simplification allowed, it can be argued that while the earlier part of the medical development of the 19th century was dominated by advances in cellular pathology, the latter part saw the dawn of another scientific revolution; bacteriology, and the establishment of the germ theory.

Although bacteria were first observed in 1675 by the Dutch microscope constructor Leeuwenhoek [17], their causative role in disease was not elucidated until the pioneering achievements by scientists like Koch and Pasteur, along with a number of their contemporaries [18]. While the pathogenic role of bacteria is an integrated part of today's common knowledge, the debate was animated within the scientific community at the time of their discoveries, in concordance with the structure of events that eventually lead to a paradigm shift [45].

One of the more prominent scientists who did not accept the germ theory was Rudolf Virchow, the father of cellular pathology [17]. As he was himself a scientific revolutionary, it would probably be unwise to discard his doubts as a mere act of ill-founded conservatism. In fact, a sceptic's case could easily be constructed: First, the same bacteria could be found in tissues, e.g. wounds, whether there were signs of disease or not. Second, the septic reaction induced by injection of bacteria into animals could be reproduced by the injection of various chemical substances [21]. Third, while bacteria could easily be cultured from the blood of animals during experimental septicaemia, this was not the case in man, where it was "evident, from the large percentage of negative results even in the severest type of disease, that the search for the specific causes of disease by this method will often prove futile" [21, 46]. So which was the actual pathogenic role of bacteria? These were issues that had to be dealt with by the scientists of the new era. They were duly resolved, chiefly by studies of host defense mechanisms, which will be discussed in a subsequent section.

Sepsis is commonly associated with bacterial infections, yet all known types of microorganisms can cause infections evolving to sepsis, exemplified by malaria (caused by a protozoan), influenza (a viral infection), and candidemia (a fungal invasion of the bloodstream). Although fungal sepsis has become increasingly common

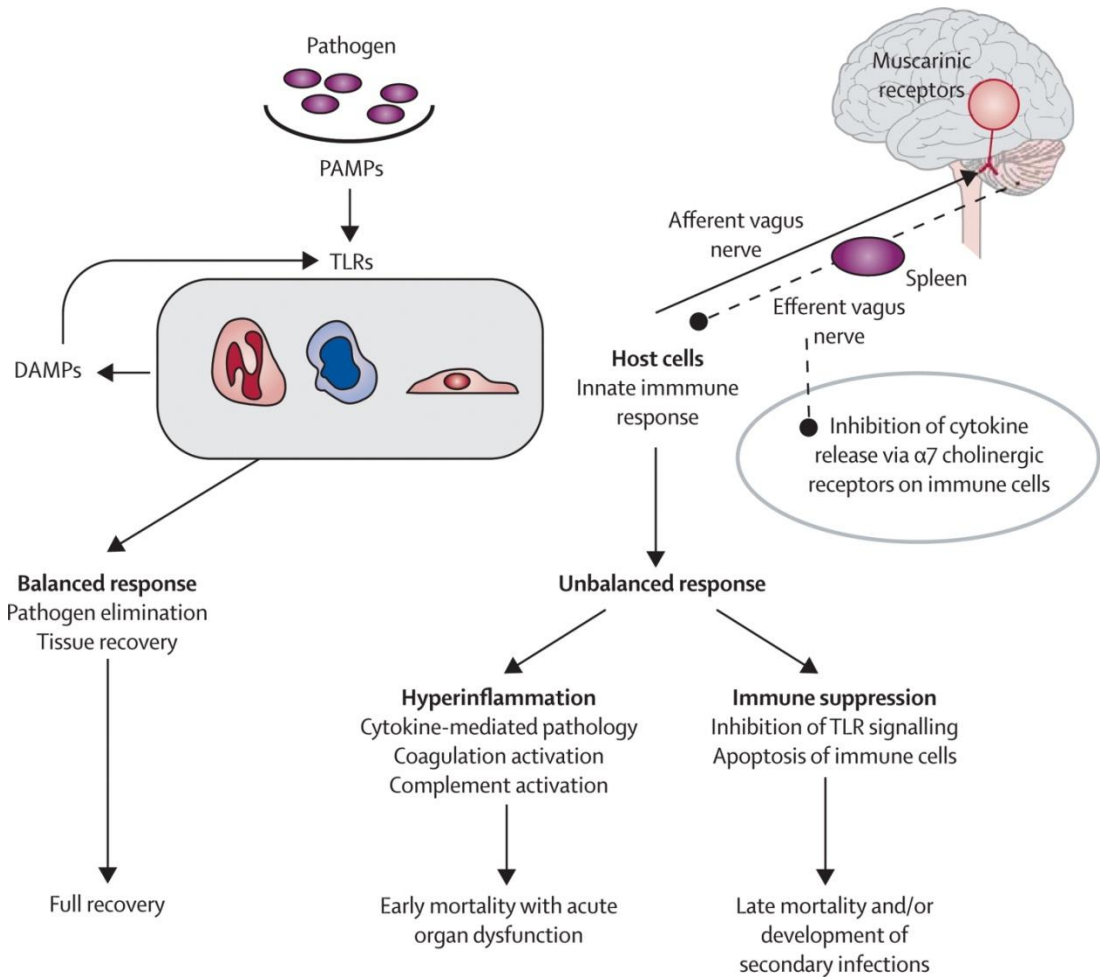


FIGURE 3. The interaction between pathogens and the host is mediated initially via an interaction between pathogen-associated molecular patterns (PAMPs) and Toll-like receptors (TLRs). This interaction can result in the release of danger-associated molecular patterns (DAMPs), which have the ability to further amplify the inflammatory response, at least in part, via TLRs. The initial inflammation activates afferent signals that are relayed to the brain; subsequent activation of vagus efferent activity inhibits cytokine synthesis via pathways dependent on acetylcholine receptors on macrophages and other cells through the cholinergic anti-inflammatory pathway (the inflammatory reflex). The resulting innate response of immune cells can result in a balanced reaction leading to pathogen elimination and tissue recovery, or an unbalanced reaction that on the one hand can lead to exaggerated inflammation and tissue injury, and on the other hand to immune suppression (reprinted from van der Poll et al. [35] with permission from Elsevier).

in the last decades [7], sepsis remains a predominantly bacterial disease [8, 13].

To the bacteria, a human host represents a nutritious environment offering colonization and prospering. This is a highly competitive milieu, in which only mechanisms that promote bacterial persistence and spread will be favoured by natural selection. The distinction between human health and disease is simply not relevant in this context; it is rather the result of mechanisms and sites chosen by the microorganism for proliferation. In general, the competition for a favourable niche within the host is to the advantage of microbes that contribute to the host's well-being (mutualists) or those that do not cause harm (commensals). Hence, commensalism and mutualism are the principal forms of human-microbe relations, established early in the life of the individual and essential to human biology [47].

A pathogen, on the other hand, faces impressive challenges. By causing harm to the host, it will activate a response that endangers its own existence. In order to become successful under such dire circumstances, pathogens use a wide array of counterstrategies. These include antiphagocytic capsules, toxins that destroy immune cells and anatomic barriers, and various refined biochemical interactions to evade or manipulate host defence mechanisms [35, 41, 48].

A number of bacterial species regularly cause sepsis in individuals with an apparently intact immune system, e.g. *Staphylococcus aureus*, *Streptococcus pneumoniae*, β -hemolytic streptococci, *Neisseria meningitidis* and various enterobacteriaceae. They are principal human pathogens that exhibit a high degree of virulence. From an evolutionary point of view, causing serious disease that threatens host viability can, literally, be a "dead end" to the microbe, unless transmission to a new host is secured in advance. The higher the virulence, the greater the need will be for efficient transmission [47]. The principal pathogenic species have solved this by a sufficiently high transmissibility, but their evolutionary success likely depends more on their ability to remain peaceful most of the time. Although feared as pathogens, their main role is that of commensal members of the indigenous microbiota, causing no or only mild symptoms of infection. The occasional cases of sepsis caused by these organisms are exceptions to the rule, representing only a minuscule part of their interaction with humans. What distinguishes them from other commensals is their inherent capacity to breach barriers and invade highly protected anatomic sites of vital importance to the host, and to persist and multiply in these environments through adaptive mechanisms.

There are, in addition, a number of commensal species that may act as pathogens solely when the delicate balance between host defense and microbiota is disrupted. In the presence of disease or medication that affects the host's defense system or microbial ecology, opportunities arise for these microbes to step forward and thrive outside their ordinary confines, thereby causing disease. They are opportunists, of which *Pseudomonas aeruginosa* and *Candida albicans* are common examples.

There is, however, no sharp demarcation between principal pathogens and opportunists when it comes to sepsis, where a more or less opportunistic behaviour can be expected from all pathogens, exemplified by the higher incidence seen in individuals of advanced age and/or with debilitating co-morbid conditions, regardless of causative pathogen [3, 8, 49].

1.4 THEORIES AND THERAPIES

The construction of modern sepsis theory is intimately associated with two parallel developments in medicine: First, the emergence and evolution of immunology, which renders the clinical picture interpretable through molecular mechanisms. Second, the establishment of intensive care treatment with the capability of lifesaving organ support, which would carry the patients through previously irreversible stages of disease and provide opportunities for novel therapies.

In the following, a brief outline of some significant steps in this evolution will be given.

1.4.1 The response

“Except on few occasions, the patient appears to die from the body’s response to infection rather than from it.”

-WILLIAM OSLER

1.4.1.1 *We are not defenseless*

As previously mentioned, the fact that blood cultures were often negative even in severely septic patients was vexing to the scientists of the late 1800s. In a review “On the present state of knowledge in bacterial science” of 1886, the author concludes that “the number of micro-organisms present in septicaemic blood of man is so small that it is frequently impossible to obtain pure cultures” and “It therefore seems natural to suppose that the micro-organisms of septicaemia are capable of producing some poisonous substance which kills the patient” [21]. Within the next few years, this hypothesis was corroborated by the discoveries of diphtheria and tetanus toxins by Roux and Yersin, scientists at the brand new Pasteur institute [18]. Diphtheria and tetanus, while terrifying and lethal diseases at the time, could not readily be termed “septic”, as their symptoms were of a different nature. Still, toxin-mediated injury later proved to be of major importance in the pathogenesis of sepsis, although not imperative for the causation [35].

The toxin discovery also became the starting point for a chain of scientific endeavors that eventually led to the establishment of a new academic discipline, sprung out of bacteriology. In 1890, Behring and co-workers could demonstrate antitoxins, substances produced in the blood of animals injected with diphtheria and tetanus bacteria, which would neutralize the toxins and cure the disease when given to symptomatic animals [18, 50].

Another German, Paul Ehrlich, made a major theoretical breakthrough when developing the serum therapy introduced by Behring. In 1897, he conceived the idea of multiple specific receptors on host cells that would multiply abundantly when triggered by harmful foreign substances, in order to neutralize them. He termed these specific receptors “antibodies”, and the notion of a specific, humoral immune response was born [18, 51, 52].

A couple of decades earlier, Ilya Mechnikov, a young professor of zoology at the University of Odessa, had been tormented by a depression with suicidal ideation. To spare his family from embarrassment, he decided to perish with style - through a scientific experiment. He inoculated himself with relapsing fever to find out whether it

was transmissible by the blood. It was, and he became severely ill, but the suicide attempt failed. Mechnikov was cured from both the fever and the depression and went on to study migratory cells challenged with foreign material. Building on his observations, he formulated the theory of phagocytosis, claiming that cells take a central part in the host defense through the active engulfment of microbial invaders, as distinct from passively being invaded, which was the dominating view [51, 52]. Mechnikov had thereby brought the concept of an unspecific, cellular immune defense into existence. He subsequently studied the function of phagocytes in various infections and, in a paper on streptococcal sepsis, distinguished between phagocytes and introduced the term “macrophage” to describe the larger of the cells [53] (the smaller “microphages” were neutrophil leukocytes).

The findings by Ehrlich and Mechnikov were, at the time, too discordant to be unified into a general theory of host defense, and instead a “humoral” and a “cellular” school was formed, leading to heated scientific disputes [52, 54]. Their theories, however, shared a common theme: when pathogens attack, the human host is not a defenseless victim – rather, we are capable of forceful countermeasures. Immunology was born, and Ehrlich and Mechnikov were jointly awarded the Nobel Prize of 1908 in recognition of their work.

Subsequent research found their theories to be complimentary; the cooperation between phagocytes and antibodies is fundamental to an efficacious elimination of microbes. (Further studies have added to the knowledge of how intricately entangled the mechanisms of the innate, unspecific “cellular” defense and the acquired specific “humoral” defense are. Still, the division of the immune system along these lines has shown to be remarkably robust over time. Whether this theoretical separation actually contributes to our understanding of the immune defense, as we know it today, could be the matter of a scientific debate. This will, however, have to take place elsewhere, as it would be beyond the scope of this thesis.)

1.4.1.2 The body at war: collateral damage

To a great extent, the landscape of current sepsis theory owes its features to the studies of one single molecule and its effects: endotoxin.

Richard Pfeiffer, a co-worker of Koch, had found that guinea pigs inoculated with cholera bacteria would die even if the bacteria had been heat-killed prior to the challenge. When using other Gram-negative bacteria he could reproduce his results, and concluded that the lethality of an infection was not necessarily related to the viability of the microorganism. He proposed that death was caused by a formerly unknown heat-stable toxic substance from the bacterial “body substance”. When realizing the potency of his discovered toxin, Pfeiffer asked with awe, “Was müssen das für Substanzen sein, die in so verschwindenden Mengen so starke Wirkungen auslösen?” [54]. He termed it “endotoxin”, to simultaneously indicate its toxic potential and the inherent association with the bacterial cell.

Today we know that endotoxin, or LPS, is a structural component of the outer membrane of Gram-negative bacteria and one of the most potent inducers of an inflammatory immune response in humans. It is this response, rather than the direct action of the LPS molecule, which may be harmful to the host in a dose-dependent manner. Minute amounts of endotoxin cause a balanced, protective response to a microbial challenge, while large doses induce shock and death [55]. Since endotoxin

elicits many of the key characteristics of sepsis, such as fever, myalgias, alterations of the leukocyte population and shock, it was enticing to assume that sepsis mortality was attributable to a similar unbalanced, exuberant immune response. It was even stated that “Our arsenals for fighting off bacteria are so powerful, . . . that we are in more danger from them than from the invaders. We live in the midst of explosive devices; we are mined.” [56].

Logically, understanding the biochemical pathways of endotoxin signaling would not only provide insights into host-microbial interaction, but also open up routes to successful sepsis therapies. Immunology thus became the principal field of research in the quest to understand sepsis, and endotoxin was an easily accessible tool for studies of a “septic” response, *in vitro* as well as *in vivo*.

1.4.1.3 A gruesome forecast: the cytokine storm

In the 1970s it was shown that macrophages were key effector cells in the host response to endotoxin [57]. Also, researchers could identify substances released from monocytes/macrophages that independently would reproduce the response to endotoxin, indicating the existence of endogenous mediators of endotoxicity [58]. In the following years, a plenitude of similar messenger molecules originating from immune cells were identified. They were normally produced during a short period in response to a stimulus (e.g. LPS), and shared the capacity to induce a range of immunomodulatory effects through the activation of specific target cells. These signal proteins were termed cytokines, and among the first to be characterized were TNF [58, 59] and IL-1 [60, 61], both powerful activators of the systemic inflammatory response seen in sepsis and endotoxemia, with overlapping and synergistic effects [33, 62]. Soon, additional “downstream” cytokines were categorized, like IL-8, which recruits and activates neutrophils (such chemotactic cytokines are often referred to as chemokines), and IL-6, an inducer of fever and the acute phase response [33] (Fig. 4).

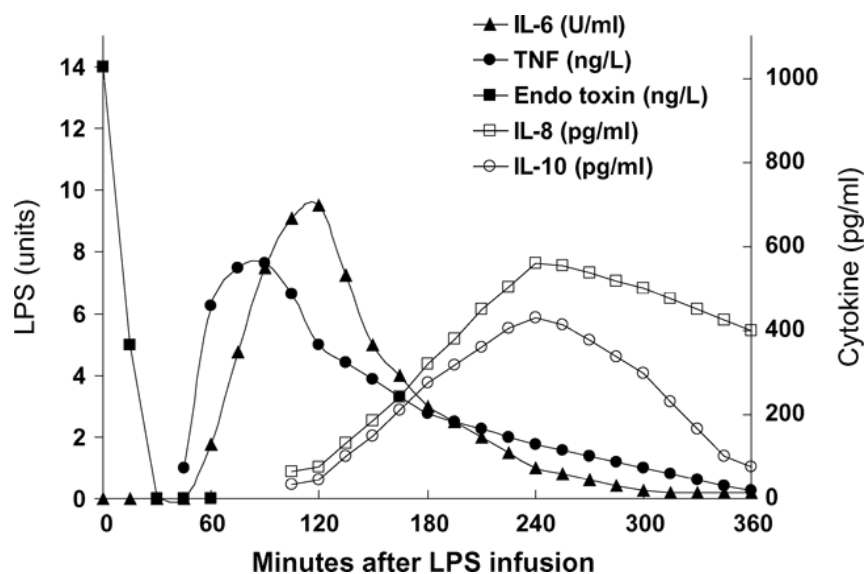


FIGURE 4. Pattern of cytokine release after endotoxin administration (reprinted from Lowry [158] with permission from Wolters Kluwer Health).

The theory of an excessive immune response responsible for death in sepsis was now strengthened by a sequence of observations: endotoxin caused profuse secretion of proinflammatory cytokines; administration of high doses of these cytokines would, like endotoxin, inflict shock and death, while lower doses caused a more moderate inflammatory response; circulating TNF was detected in sepsis patients and an association between high levels and a poor outcome was demonstrated [62, 63]. Finally, antibody-mediated blockade of TNF protected laboratory animals from the lethal effects of endotoxin and sepsis [64, 65]. This was very close to a definite proof of principle: fatal sepsis was caused by an uncontrolled release of proinflammatory cytokines, leading to a cascade of injurious events resulting in irreversible damage to vital functions. The term “cytokine storm” was established to describe this process [66], and the stage was set for the introduction of innovative therapies striving to temper an immune system gone berserk.

1.4.2 In pursuit of new therapies

*“And thus the native hue of resolution
Is sicklied o’er with the pale cast of thought”*

- WILLIAM SHAKESPEARE, HAMLET

1.4.2.1 From bench to bedside - a dead end?

There were, back in the early 1990s, freshly acquired insights into the immunological pathways of sepsis. There were new substances, like monoclonal antibodies, ready to counteract the effects of specific proinflammatory mediators, in order to reverse the cascade of events that caused organ failure. Also, there were new sepsis definitions that would allow enrolment of large patient cohorts in clinical trials. Finally, there was an enthusiastic pharmaceutical industry, braced by promising results from animal and early phase clinical trials. This was the time for large-scale randomized placebo-controlled trials.

Among the first modulators of a proinflammatory response to reach phase III sepsis trials were inhibitors of endotoxin, TNF and IL-1. While some of the studies could report benefits in subgroups of patients the overall results were disappointing, failing to demonstrate reductions in sepsis mortality [67-69] (for the pharmaceutical industry, some of the disappointment was probably mitigated by the dramatic success of TNF-inhibitors in the treatment of chronic inflammatory conditions like rheumatoid arthritis and Crohn’s disease, now a billion dollar market).

Meanwhile, groundbreaking discoveries in the field of innate immunity were continuously reported. It was now recognized that the SIRS-inducing, proinflammatory response was soon to be followed by a balancing response, mediated by anti-inflammatory molecules like IL-10 (Fig. 4) and IL-1ra. Also, from previous experiments it was known that exposure to endotoxin, *in vitro* and *in vivo*, led to a drastically diminished immune response to further LPS challenges. This state was called “endotoxin tolerance” and was thought to protect the organism from the harm of repeated endotoxemia, by a “reprogramming” of leukocytes and a reduced production of proinflammatory mediators [70, 71]. A similar down-regulation in monocytes, characterized by a reduced expression of the antigen presenting receptor HLA-DR, together with an inhibited capacity to secrete proinflammatory cytokines, was also demonstrated in human sepsis [72, 73]. Taken together, these data moderated the view

of sepsis as a predominantly hyperinflammatory state. Indeed, it could not be excluded that the aggressive anti-inflammatory treatments given in sepsis trials could have aggravated an already immunosuppressed condition in large groups of patients, thereby possibly eradicating a positive signal from groups that had benefitted from the therapy. This led to some self-critical reflection, a reassessment of the sepsis hypothesis, and the invention of another acronym, CARS, from some of the authors of the original ACCP/SCCM criteria [74].

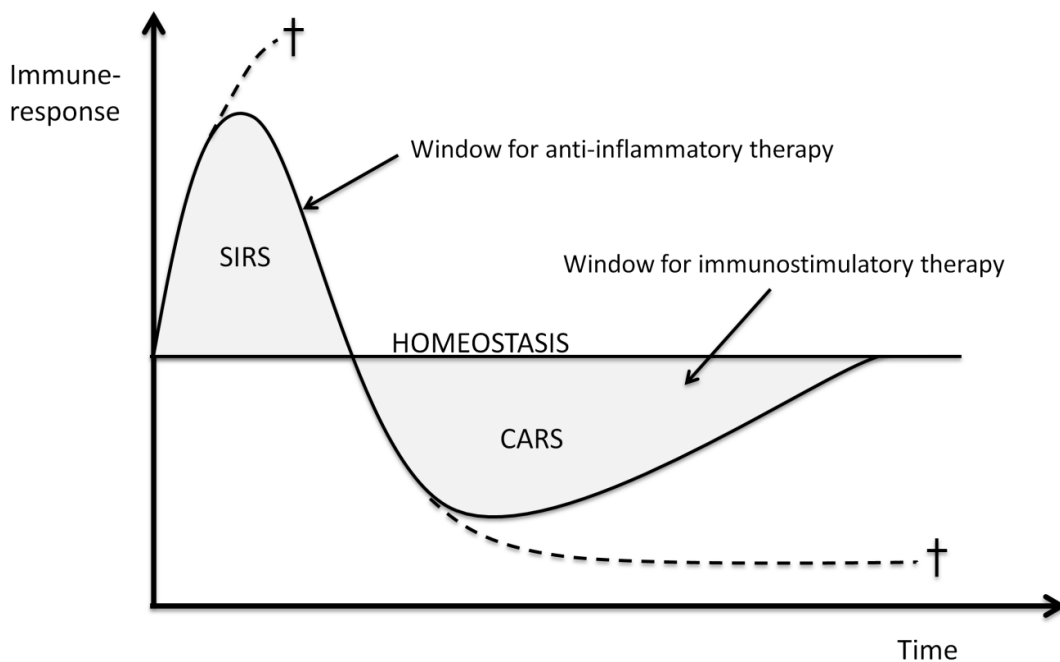


FIGURE 5. Imagined pro- and anti-inflammatory systemic immune responses over time during severe sepsis/septic shock. The dotted lines represent unbalanced responses leading to death. SIRS; systemic inflammatory response syndrome. CARS; compensatory anti-inflammatory response syndrome.

1.4.2.2 CARS – a deadly vehicle?

“Compensatory anti-inflammatory response syndrome” or CARS was one of several terms given to the immunosuppressive phase detected in sepsis. Like SIRS, CARS was believed to be potentially detrimental when unchecked, presumably through an impeded clearance of microbes and increased susceptibility to opportunistic secondary infections. The inflammatory response model was modified accordingly, giving rise to speculations on novel therapeutic strategies, where immunostimulation by proinflammatory agents could play a role (Fig. 5). There was, however, also a furthered understanding of the complexity of the disease process [72, 74, 75]. Targeting appropriate patient populations, likely to benefit from future immunomodulatory interventions, whether pro- or anti-inflammatory, would necessitate reliable markers for the monitoring of patients’ inflammatory status [27]. Surprisingly, this contention was largely ignored by clinical investigators. The search for a “magic bullet” which supposedly would cure a large generic sepsis population, only defined by organ failure and SIRS criteria, has consequently continued unabated during the last decades. Until now, only one product indicated for the treatment of sepsis, drotrecogin alpha

(activated protein C, an anticoagulant mediator), has reached the market. It was recently withdrawn by the manufacturer due to failure to reproduce the initial positive results [12]. This persistent lack of success has caused an ongoing debate, where some voices advocate a moratorium on clinical sepsis trials until better patient definitions are achieved [76], while others suggest promising new candidates for successful immunomodulation, albeit with a more personalised approach [43].

1.4.2.3 Back to basics

At the turn of the millennium, encouraging advances seen in other high risk conditions like multi-trauma, myocardial infarction and stroke were conspicuously lacking in the field of sepsis, where mortality remained unacceptably high [77]. In the absence of a novel magic-bullet-therapy, attention turned to existing health care practices.

1.4.2.3.1 Supportive treatment

From animal sepsis models it was well known that early interventions were more likely to be successful, a treatment strategy which had also significantly reduced morbidity and mortality in acute cardiovascular disease and trauma. The term “golden hour” was used in trauma care to describe a critical period where timely treatment made a difference between life and death. Perhaps it was then only proper that a group of emergency care physicians, familiar with the “golden hour” concept, translated it into sepsis treatment. In their 2001 landmark report on an aggressive resuscitation approach termed “early goal-directed therapy” (EGDT), they aimed to attain a number of predetermined hemodynamic goals during the patients’ first 6 hours in the emergency department (ED) through the employment of fluids, blood and vasoactive drugs. The ultimate goal was to reverse global tissue hypoxia, and the investigators could demonstrate a decrease in mortality from 46.5% to 30.5% in patients with severe sepsis and septic shock [78]. This impressive result attracted wide attention and shifted, at least partly, the focus of sepsis treatment from the intensive care unit (ICU) to the ED.

While the mortality reduction undoubtedly was notable, the overall mortality rates presented in the EGDT study were extremely high regardless of treatment group, thus representing a profoundly ill study population. For instance hypoperfusion, as illustrated by mean blood lactate levels, was 7.3 mmol/L in the subjects, a greatly elevated value even in regard to the severe sepsis limit at 3 mmol/L. Although a severely ill population has the advantage of enabling statistically significant differences in hard outcome variables like mortality, it also follows that results may not be generalizable to, in this case, a wider population of sepsis patients presenting to the ED. Indeed, subsequent reports indicated that patients qualifying for EGDT would only represent a few percent of these [79, 80]. It seemed like the early treatment protocol was actually given to patients in an advanced, late stage of disease. Obviously, detection and intervention at an earlier disease stage held considerable promise for further reduction of sepsis morbidity and mortality.

1.4.2.3.2 Antimicrobial therapy

When considering that sepsis was postulated a host response to infection, it is perhaps not surprising that it was the area of immunology which attracted most

research efforts in the search for new therapies. Also, since the development of modern sepsis theory was closely connected to the development of intensive care and the emerging capability of lifesaving organ support [81], many of the most influential scholars of the field were rooted in intensive care. One might speculate that this circumstance caused some attention to be channeled away from the treatment of invading microbes toward the treatment of endangered organs. There was, in any case, a notion that antibiotics, although indispensable in treating the underlying infection, would not alter the effects of an exuberant inflammatory response, which would require specific therapy [23]. The contribution of antibiotic therapy to a reduced mortality had been made decades earlier, and was now taken for granted, included as a fixed variable in the total sepsis risk equation.

However, there were a number of factors which helped to reawaken interest in antimicrobial therapy. First, after numerous failed immunomodulator trials, this was a period of reflection and a return to basic treatment strategies. Second, there were recent studies confirming a negative impact on survival when inappropriate antimicrobial therapies were given to critically ill patients [82-84]. Third, it was by now evident that successful sepsis treatment was threatened by the rising global health problem of antimicrobial resistance. Finally, in a seminal article Kumar et al. published compelling evidence of the importance of timely administration of appropriate antimicrobials to patients with septic shock. Each hour delay in accomplishing administration of adequate antibiotics led to an average decrease in survival of 7.6%, and time to initiation of effective therapy was the single strongest predictor of outcome [30]. This study had some significant implications. Septic shock was established as a “golden hour” diagnosis where prompt institution of appropriate antimicrobials was a therapeutic cornerstone. Also, it became apparent that effective halting of bacterial growth was a *sine qua non* for the successful reversal of a deleterious host response.

1.5 THE CLINICIAN'S CONDITION

The sepsis diagnosis is based on an imperfect combination of vital physiological and biochemical abnormalities and cultures, none of which *per se* is specific enough for a definitive diagnosis. The clinical picture is notoriously variable and share numerous signs and symptoms with several critical conditions, making it particularly susceptible to biased decision making. Among critically ill sepsis patients, a misjudgment earlier in the continuum of care is a common finding, and a recurring topic of intraprofessional discussions and morbidity & mortality rounds. It is obvious that clinicians, experienced or not, need to corroborate their clinical decisions with objective data when it comes to sepsis.

Results from cultures are not available until several days and do not contribute to clinical decision-making in the acute situation. Under such conditions, additional information beyond that which is obtainable from clinical examination is precious. For further insight into pathogenesis and prognosis, numerous molecules involved in the systemic response to infection have been proposed as biomarkers of sepsis [85]. Most of these are still in the experimental state, but a few, like CRP and WBC, have been routinely employed in acute care settings for many years, while others, like procalcitonin (PCT), have been introduced only recently. LBP, IL-6, soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) and heparin-binding protein (HBP) are examples of mediators of the innate immune response that have been suggested as

sepsis markers, but have not yet made their way into clinical practice [81, 86]. A brief description of biomarkers relevant for this thesis is presented below.

1.5.1 Biomarkers of relevance for this thesis

1.5.1.1 WBC and neutrophils

White blood cells or leukocytes are protagonists of the inflammatory response and were among the first immune cells to be described. Neutrophils form the major part of circulating leukocytes and are promptly recruited to an inflammatory site in response to infection or tissue damage. Neutrophil demargination and bone marrow mobilization result in rapidly increased and readily measurable WBC and neutrophil counts, emblematic of an acute systemic inflammation. This effect, however, is a general inflammatory reaction and not specific for infections.

1.5.1.2 C-reactive protein

CRP, one of the first reported acute phase proteins, was described in 1930 as a substance in the sera of pneumonia patients which precipitated the C-polysaccharide of *Streptococcus pneumoniae* [87]. Roughly half a century later it was widely adopted as a marker of infection in Swedish health care settings. Primarily, CRP production follows upon IL-6 activation of hepatocytes. It binds to various structures, among them bacterial surfaces, damaged cells and complement. CRP thereby activates the complement system and facilitates bacterial opsonization. The fact that plasma levels may increase more than a thousandfold within a few days after immune activation makes CRP a suitable biomarker for acute care situations. As an acute phase protein, CRP production is associated with a number of inflammatory conditions, including chronic inflammatory diseases, trauma and pancreatitis. Hence, CRP is not a specific marker of infection [33, 88].

1.5.1.3 Procalcitonin

PCT as a marker of infection and sepsis was not described until 1993 [89]. A precursor of calcitonin, PCT is predominantly produced in the thyroid under homeostatic conditions, but may be ubiquitously and rapidly expressed in response to various inflammatory challenges. While increased levels of PCT have been reported for numerous conditions like trauma, malignancies and pancreatitis, it is suggested to be more specifically associated with bacterial infections of a systemic nature than other biomarkers. Elevated plasma levels of PCT can be detected within hours after an insult, with peak values as soon as after 12-24 h, which is earlier than for CRP. This kinetic profile is considered favourable in the diagnostics of infections at risk of rapid progression [81, 90, 91].

1.5.1.4 Lipopolysaccharide binding protein

While LPS, component of Gram-negative bacteria and a potent PAMP, had been extensively studied for decades, the response protein LBP was not described until 1986 [92]. LBP is present in the circulation at low levels under normal conditions, but increases during the acute phase reaction with a kinetic profile similar to that of CRP. It is, like CRP, an acute phase protein, mainly synthesized by hepatocytes in response to

IL-1 and IL-6. Since LBP binds to free LPS, it has been proposed as a marker of sepsis and Gram-negative infections [33, 81, 93].

1.5.1.5 Interleukin 6

Cytokines are key regulators of numerous inflammatory responses, and increased plasma levels can be detected within hours of an inflammatory insult (Fig. 4). Most inflammatory cytokines have been reported in association with sepsis. IL-6, an important inducer of the systemic inflammatory response, is mainly responsible for the initiation of fever and the acute phase response. As an “upstream” mediator it is synthesized and detectable more rapidly than the proteins of the acute phase response, thus making it a potentially valuable marker in the earlier stages of sepsis. High levels of IL-6 have also repeatedly been reported in association with poor prognosis and organ dysfunction [33, 94-97]. Nonetheless, like most mediators of the innate immune response, IL-6 can also be triggered by various non-infectious conditions.

1.5.1.6 Cytokine profiling

Although circulating cytokines have been measured in association with sepsis and SIRS for many years, no specific cytokine or combination of cytokines has emerged as a reliable marker fit for clinical practice. This may in part depend on the influence of circulating soluble receptors and various interactions with plasma proteins, obscuring associations of significance for the disease process. For this reason, we speculated that an alternative methodological approach could yield more accurate results.

1.5.2 The antibiotic dilemma

Since administration of adequate antibiotics is urgent, treatment has to be given empirically, before culture results are available. The diagnostic uncertainty is compensated for by the usage of one or more broad-spectrum antibiotics to patients with presumed severe sepsis/septic shock, an approach which also may propel the process of escalating antibiotic resistance among bacteria. Thus, on the one hand the risk of a deteriorating patient, on the other hand the contribution to growing resistance. The second part of this dilemma is easily disregarded at the patient’s bedside, which is one of the obvious reasons for the precarious current situation. When there is little margin for error, as in the case of a critically ill patient, the scales are tipped in favor of unselective, broad antimicrobial therapy. While point prevalence studies indicate that about a third of the patients in a general hospital population are under antibiotic treatment [98], this fraction is more than twice as high in the ICU setting [99, 100], which has been called an “epicenter for the acquisition and dissemination of antibiotic resistance in bacterial pathogens” [101].

This predicament could, however, be alleviated by better tools for appropriate selection of patients with evidence of infection, preferably before progression to a more severe stage of disease. We hypothesized that it would be possible to define such a risk population through a systematic collection of key clinical variables and biochemical data in the early hospital course of patients admitted from the ED with a suspected severe infection (Fig. 6).

Additionally, we imagined it feasible to monitor the inflammatory response in sepsis by employing the enzyme-linked immunospot (ELISpot) technique. As opposed to

determining the amount of circulating cytokines, ELISpot measures cytokine secretion at the very source, the individual cell. We hypothesized that this “immunological snapshot” of cytokine secreting cells could provide a more relevant illustration of the association between cytokine release and disease.

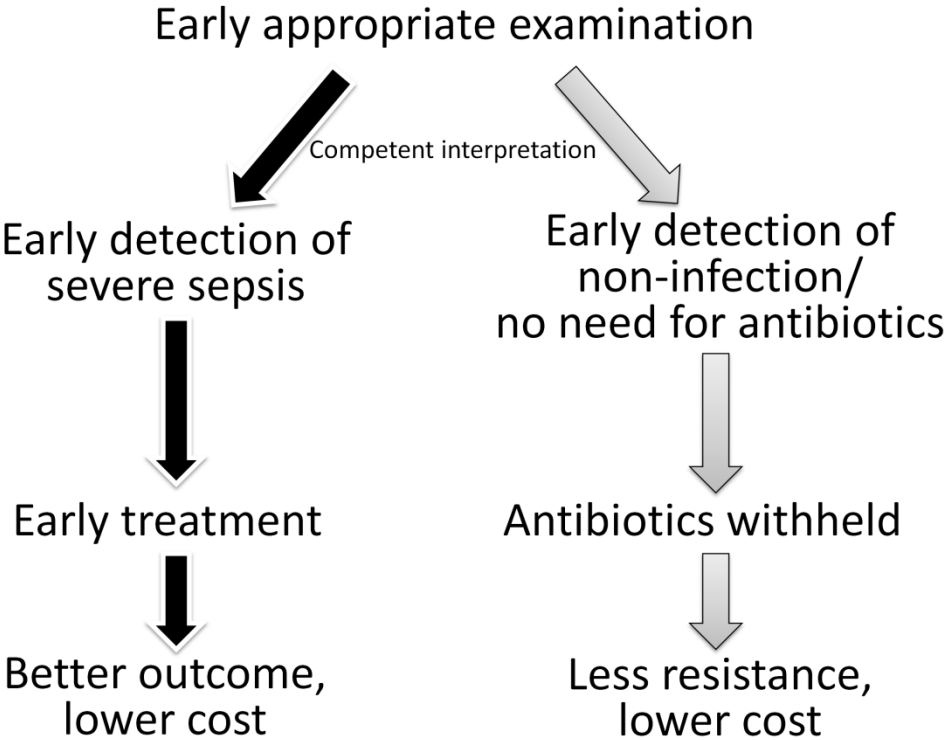


FIGURE 6. Schematic illustration of an ideal distribution of ED patients after appropriate examination and competent interpretation of available markers of infection

2 AIMS

2.1 GENERAL AIMS

- A. To assess the prevalence and clinical impact of severe sepsis and the diagnostic value of currently available clinical and biochemical markers in a patient population with a suspected serious infection.
- B. To increase knowledge of cytokine production at the cellular level in response to sepsis and endotoxin through the introduction and evaluation of the ELISpot technique in this context.

2.2 SPECIFIC AIMS

- Paper I To evaluate a number of clinical and laboratory variables measured early in the hospital course of patients admitted with a suspected severe infection for their ability to identify conditions that require antibiotic treatment. Secondary aims were to determine the potential of the same variables to detect bacteraemia and severe sepsis.
- Paper II To assess the prevalence and clinical impact of severe sepsis in a selected ED population with a clinical suspicion of severe infection and, in addition, to investigate the association of SIRS criteria with severe sepsis and a subsequent critical course.
- Paper III To examine cytokine secretion from circulating leukocytes in sepsis, in particular in relation to severity and stage of disease.
- Paper IV To investigate the cytokine release of different leukocytes in response to endotoxin based on the number of producing cells.

3 MATERIALS AND METHODS

3.1 SUBJECTS

3.1.1 Paper I-II

A total of 404 patients at risk for a severe infection who were admitted from the EDs at the Karolinska University Hospital (Solna and Huddinge sites) to the Department of Infectious Diseases were enrolled prospectively from August 2004 through October 2005, using convenience sampling. The attending clinicians' decision to order blood cultures and to admit the patient to the ward served as markers for a suspicion of a severe infection. Patients with prior participation in the study or age below 18 years were excluded.

The results presented in paper I and II were derived from this patient cohort.

3.1.2 Paper III

This was a prospective observational study of 32 patients admitted to the Karolinska University Hospital (Solna and Huddinge sites) from July 2009 through July 2010, using convenience sampling. Patients who were admitted to the hospital and presented with signs of sepsis according to consensus criteria [23] were eligible. Exclusion criteria were age below 18 years and leukopenia due to hematological disease or chemotherapy. Healthy volunteers (n=30) from the hospital staff served as controls. A majority of the patients (26/32; 81%) were enrolled from ICUs.

Nine healthy volunteers were also administered LPS for an experiment of human endotoxemia.

3.1.3 Paper IV

Healthy human volunteers exclusively served as blood donors in this methodological study.

3.2 METHODS

3.2.1 Data collection (paper I-III)

3.2.1.1 Paper I-II

Vital sign data were recorded on admission to the ED and every 4 h for the first 24 h at the hospital on a 24h/7d basis: body temperature, heart rate, blood pressure, respiratory rate, oxygen saturation (pulse oximetry), urine output, signs of confusion or decreased alertness.

Age, sex, and significant comorbidities (malignancy, chemotherapy, liver disease, congestive heart failure, renal failure, chronic obstructive pulmonary disease, diabetes mellitus, neurological disorder, transplant, HIV, and substance abuse) were recorded as well as time of arrival to the ED, the time of initiation of antimicrobial therapy, length of stay at the hospital and 28-day mortality.

Blood was drawn on admission from all patients for cultures (at least 10 ml each for aerobic and anaerobic cultures), analysis of hemoglobin (Hb), WBC, neutrophil count, platelet count, CRP, PCT, IL-6, LBP, lactate, prothrombin time (INR), D-dimer, albumin, serum creatinine, urea, bicarbonate, and bilirubin. Results from all biochemical analyses were available to the treating clinician, with the exception of PCT, IL-6 and LBP. Other cultures and analyses were left to the discretion of the treating physician.

3.2.1.2 Paper III

Data of relevance for diagnoses and severity stratification were retrieved retrospectively through chart reviews and from the Patient Database Management System (Clinisoft, GE Healthcare) of the participating ICUs. Blood for subsequent ELISpot analysis was obtained from patients and controls on inclusion.

3.2.2 Diagnostic criteria and severity stratification (paper I-III)

We used criteria adapted from sepsis consensus definitions [23, 29] to stratify patients according to sepsis severity. To render possible an evaluation of the presence of SIRS in relation to severe sepsis in paper II, SIRS was not considered a prerequisite for this diagnosis. Severe sepsis was thus defined as a state with organ dysfunction/s induced by an acute infection within 24 h of hospital stay in paper I-II. In paper III most patients were treated in the ICU and septic shock was defined as severe sepsis with hypotension requiring vasopressor therapy or refractory to adequate volume resuscitation for >1 hour. We also employed the traditional SIRS criteria for the sepsis diagnosis in this study. For further severity stratification we utilized the Sequential Organ Failure Assessment (SOFA) and Acute Physiology and Chronic Health Evaluation II (APACHE II) scores according to the Swedish Intensive Care Registry guidelines [102].

Final main infectious diagnoses, settled by attending physicians through regular diagnostic procedures, were audited independently and retrospectively by two of the authors (P.G-J. and B.G.) with the use of predefined diagnostic criteria collected from widely published guidelines or similar authoritative sources. Patients with an indeterminable diagnosis were exempted from further analyses.

3.2.3 Laboratory methods (paper I-IV)

Accredited hospital routine methods were used throughout for laboratory analyses. Exceptions are listed below.

3.2.3.1 PCT, IL-6 and LBP (Paper I)

For PCT, IL-6 and LBP discussed in paper I, serum was stored in frozen aliquots at -70°C for later analysis. PCT was analysed with an immunoluminometric assay (BRAHMS AG Berlin, Germany), while IL-6 and LBP were analysed with a chemoluminescence immunoreaction method (Imulite, SPC Scandinavia AB, Mölndal, Sweden).

3.2.3.2 *The ELISpot assay (paper III-IV)*

The ELISpot assay is a highly sensitive immunoassay employed for a variety of applications to study immune cell responses. Since the ELISpot assay is between 20 and 200 times more sensitive than conventional enzyme-linked immunosorbent assay (ELISA) and allows for detection of cytokines at the single cell level, it is particularly suited for cytokine detection in small populations of activated cells and may reflect in vivo conditions more accurately. In comparison with ELISA, ELISpot measures frequencies of secreting cells as opposed to the total amount of released cytokines. Furthermore, the ELISpot assay is dissimilar to techniques using mRNA and intracellular staining, in that it exclusively detects actually secreted cytokines.

ELISpot was used to investigate cytokine secretion (TNF, IL-6, GM-CSF, IL-1 β , IL-12p40, IL-10) from separated leukocytes in paper III, and from PBMC and PMN in paper IV (IL-1 β , IL-6, IL-8, IL-10, IL-12, TNF, GM-CSF, MIP-1 β ; in addition IFN- γ , perforin and granzyme B were tested in PMN exclusively).

Ninety-six-well membrane plates were pre-coated with desired specific capture antibodies, followed by the introduction of separated cells at predefined cell concentrations to wells containing medium, with or without LPS, and incubated for 20 hours at 37°C in 5% CO₂. After incubation, cells were removed by phosphate-buffered saline (PBS) washing, and biotinylated detection antibodies binding to captured cytokines were added. Subsequently, a streptavidin-alkaline phosphatase conjugate was added, which in combination with the final addition of a substrate formed a colored precipitate, constituting a visual spot corresponding to one single secreting cell. Counting of spots was performed in an ELISpot reader system. The principal steps of the assay procedure are illustrated in figure 7.

3.2.3.3 *Luminex assay (paper III)*

Plasma levels of the investigated cytokines were analyzed using a kit based on the multiplex Luminex technology according to the manufacturer's instructions.

3.2.3.4 *ELISA (paper IV)*

In order to compare ELISpot results to conventional ELISA, LPS-stimulated granulocytes were incubated for 20 hours, using uncoated ELISpot plates. Supernatant concentrations of IL-6, TNF and IL-1 β were subsequently analyzed in pre-coated ELISA kits according to the manufacturer's instructions.

3.2.3.5 *Isolation of cells (paper III-IV)*

Freshly collected blood from study subjects served as the source of cells.

Peripheral blood leukocytes (paper III) were separated by the mixing of equal volumes of blood and a 2% dextran PBS solution, where erythrocytes were allowed to sediment. The remaining leukocyte buffy coat was collected, and cells were counted and centrifuged. The supernatant was removed and stored for later analysis of cytokines, while the leukocyte pellet was resuspended in cell culture medium, diluted to desired cell concentrations and incubated in ELISpot plates.

PBMC (paper IV) were isolated by the mixing of equal volumes of blood and PBS, where the mixture was layered on Ficoll-Paque PLUS and centrifuged. The PBMC

layer was collected and washed twice with cell culture medium. Cells were counted and diluted to desired concentrations before entering ELISpot plates.

Monocytes were, for one experiment, depleted from the PBMC fraction by applying anti-human CD14 magnetic particles. After incubation, cells were suspended in cell

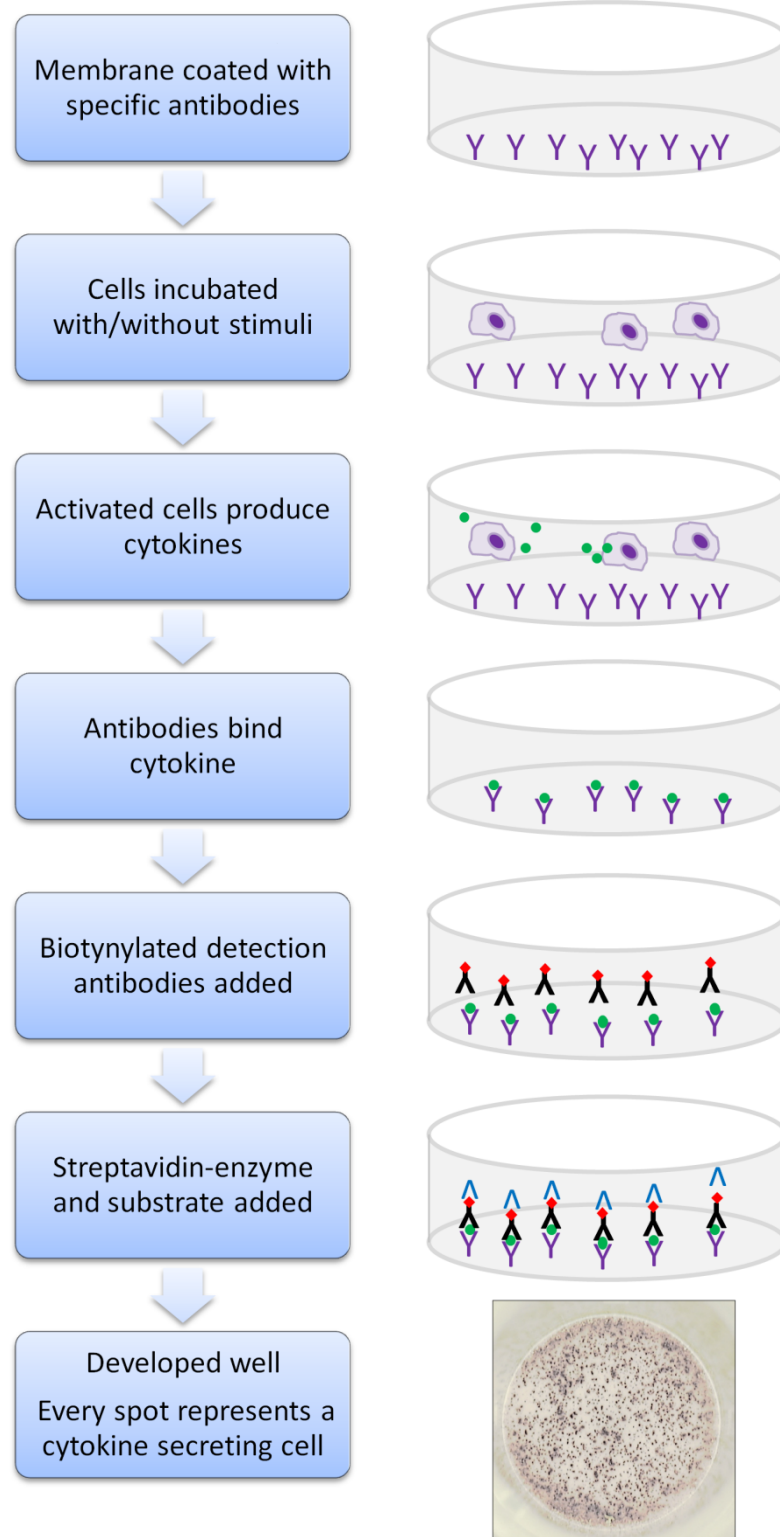


FIGURE 7. Principal steps of the ELISpot assay procedure

culture medium and placed in a magnet device according to the manufacturer's instructions. The unbound cell fraction, depleted of monocytes, was retrieved and washed. The depletion procedure was then repeated once.

PMN (paper IV) were isolated by using the erythrocyte-granulocyte pellet after Ficoll separation. After resuspension in PBS to original blood volume, the sample was layered on Polymorphprep. After centrifugation the PMN fraction was removed and mixed with PBS. Cells were washed twice in cell culture medium and contaminating erythrocytes were depleted by hypotonic lysis. Remaining PMN were counted and diluted to desired concentrations. Light microscopy and flow cytometry was employed to determine the purity of the preparation, which was found to be > 98%.

Cell viability was controlled via trypan blue exclusion for all extracted cell types.

3.2.4 Statistics (paper I-IV)

To compare quantitative variables the Mann-Whitney *U*-test and the unpaired Student's *t*-test were used, as appropriate. The χ^2 -test or Fisher's exact test were applied for categorical variables. Correlation analysis (paper III) was performed by Spearman's test. For the comparison of naïve and LPS-stimulated cells in paper IV, Wilcoxon's signed rank test was used. Additionally, for paper I, binary logistic regression was used for multivariate analyses and receiver operating characteristic (ROC) analysis was employed for the evaluation of diagnostic performance of individual markers.

A significance level of 0.05 was used throughout.

3.2.5 Ethics (paper I-IV)

All studies were approved by the Regional Ethical Review Board at the Karolinska Institute.

4 RESULTS AND DISCUSSION

4.1 PAPER I-II

After ED triage the patients of this study were directed to the infectious disease physician on duty, due to a suspected infection. Patients were subsequently admitted to the infectious disease department with a preliminary diagnosis of an infection-induced illness with a severity that required hospitalization. In the absence of microbial confirmation the attending physician relied on the clinical presentation, vital signs, and a set of biomarkers for diagnostic and therapeutic guidance. In this cohort, patients with a non-infectious final diagnosis evidently presented with symptoms and signs consistent with an acute infectious disease.

In paper I, we evaluated a selection of vital signs and biochemical variables for their accuracy to identify a number of clinically relevant conditions within this highly selected population. With the exception of PCT, LBP and IL-6, results from biochemical analyses were accessible to the admitting clinician.

In paper II, we assessed the prevalence and clinical impact of severe sepsis in this population and the association of SIRS criteria with severe sepsis and a subsequent critical course.

Patients' characteristics and distribution within the investigated conditions are reported in table 1 and figure 8. In the following, the term "organ dysfunction" may refer to both hypotension, hypoperfusion and specific organ dysfunctions according to the definition, unless otherwise indicated.

TABLE 1. Patient characteristics paper I-II (N=404)^a

	Age; median (range)	61 (18-100)
	Gender male/female	201 (49.8) / 203 (50.2)
	Location prior to admission	
	Long term care facility	29 (7.2)
	Home	375 (92.8)
	Underlying chronic conditions	157 (38.9)
	Subject to intensive care	18 (4.5)
	Mortality at 28d	11 (2.7)
	Length of stay, days; median (range)	4 (1-96)
	Infection requiring antibiotics ^b	286 (70.8)
	Bacteremia	68 (16.8)
	Severe sepsis	156 (38.6)
	Final diagnosis	
	Respiratory tract	139 (34.4)
	Urinary tract	63 (15.6)
	Skin / soft tissue	43 (10.6)
	Gastrointestinal	27 (6.7)
	Miscellaneous bacterial	45 (11.1)
	Nonbacterial ^c	27 (6.7)
	Noninfectious ^d	26 (6.4)
	Unevaluable	34 (8.4)

^a Data are no. (%) of patients, unless otherwise indicated.
^b pneumonia *n*=128, urinary tract *n*=63, skin/soft tissue *n*=43, abdominal focus *n*=12, endocarditis *n*=6, orthopedic infections *n*=5, catheter related infections *n*=5, neutropenic fever *n*=5, gastrointestinal *n*=5, bacterial meningitis *n*=3, dental infection *n*=3 and 2 each of bloodstream infection, otitis media, upper respiratory tract and rickettsial infection
^c viral *n*=25, malaria *n*=2
^d malignancy *n*=5, pulmonary embolism *n*=4, systemic diseases *n*=3, musculoskeletal disease *n*=4, and 1 each of cerebral infarction, myocardial infarction, chronic obstructive pulmonary disease, Sweet's syndrome, reactive pericarditis, allergic skin reaction, reactive arthritis, postoperative fever, heart failure and postinfectious asthenia

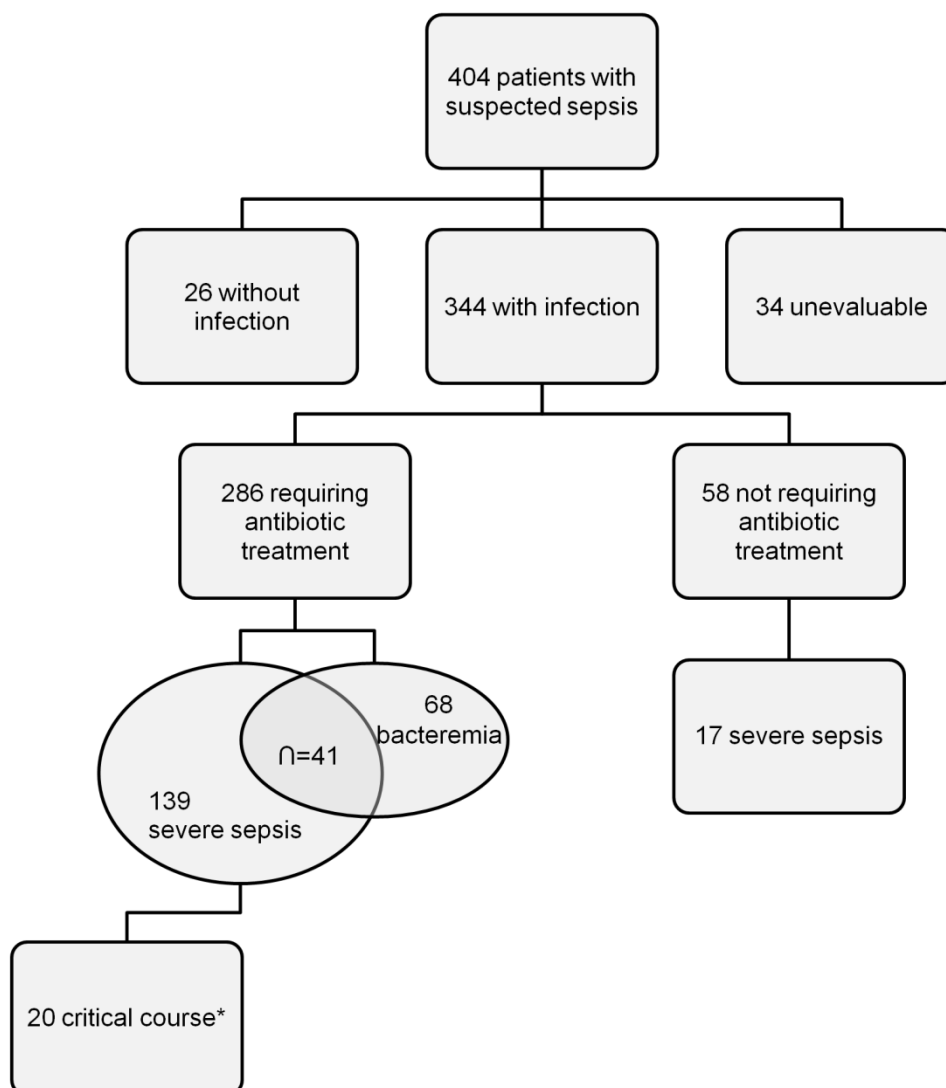


FIGURE 8. Infections and severe sepsis in enrolled ED patients of paper I-II.
 *Intensive care treatment ($n=14$) and/or death ($n=8$) within 28 days

4.1.1 The vitals

Patients at risk for imminent deterioration require appropriate interventions urgently. Under such circumstances, instantly available information is most valuable, preferably at bedside. The four classic vital signs, temperature, heart rate, blood pressure and respiratory rate (RR), belong to this category. They are easily controlled repeatedly with a minimum of instrumentation and discomfort to the patient. Their contribution to a reliable assessment of fundamental physiological functions make them part of most hospital monitoring and triage systems [103-105]. In addition to the classic vital signs, we also controlled oxygen saturation (pulse oximetry), urinary output, and signs of confusion or decreased alertness.

The vital signs are not disease specific, an advantage when developing severity assessment systems based on more generic groups of patients, but a potential drawback for a diagnostic tool. For this reason it was interesting that, within this selected

population, RR actually contributed significantly to the correct classification of infections requiring antibiotic therapy and, also bacteremia. When patients with pneumonia were excluded from the analysis, this result was not changed. This was contrasting with the other registered vital signs, of which only the initial mean arterial blood pressure (MAP) was associated with bacteremia in the univariate analysis, an association which was lost in the multivariate analysis. Although sensitivity and specificity was modest, the highest registered RR during the first four hospital hours yielded a slightly larger area under the ROC curve (AUC) than the RR at presentation, which supports the benefit of repeated measurements. Median RR for patients in need of antibiotic therapy and bacteremia was, respectively, 24 and 24.5/min (Table 2).

Temperature, heart rate, and RR also constitute three of the classical SIRS parameters, together with WBC (Box 1). Meeting the SIRS criterion of a RR >20 was significantly associated with the diagnosis of severe sepsis as well, which was not seen for the temperature and heart rate parameters. The median RR for patients with severe sepsis was 24/min, equal to that of patients with bacteremia and an infection requiring antibiotics.

In severe sepsis, respiratory dysfunction has been shown to be an early and frequent sign of prognostic importance [106-109]. The cytokine driven inflammatory response augments body temperature and the global metabolic demand, which together with local effects within the respiratory system induces an increase in RR and tidal volume. Moreover, hypoperfusion induces acidosis, which also increases alveolar ventilation [110, 111]. Several scoring systems intended for various populations have included a RR of ≥ 30 as a cut-off value indicative of severe disease [112-114]. Logically, sepsis associated respiratory derangements occur on a continuous scale. Our results suggest that, for an ED population at risk of developing a severe infection, an elevated RR merits attention also at more moderate levels. Interestingly, a RR of 24/min has been suggested as a cut-off level for critical illness, based on data from various hospital populations [110].

TABLE 2. Variables independently predicting an infection requiring antibiotics or bacteremia in a multivariate logistic regression model

Indicator	Need for antibiotics		Bacteremia		
	p	Median (IQR) with / without antibiotic need	Indicator	p	Median (IQR) with / without bacteremia
WBC ($\times 10^9/L$)	0.001	12.7 (9.2-17.2) / 9.0 (7.0-12.6)	CRP (g/L)	0.006	231 (81-352.5) / 131 (49-228)
Hemoglobin (g/dL)	0.002	13.0 (11.6-13.9) / 13.5 (12.5-14.4)	Respiratory rate	0.008	24.5 (20-31.5) / 22 (18-26.5)
Respiratory rate	0.005	24 (20-28) / 20 (17-24)	PCT ($\mu g/L$)	0.008	4.1 (0.9-17.2) / 0.3 (0.1-1.9)
CRP (g/L)	0.010	167 (72-273) / 60 (18-157)	Bilirubin ($\mu mol/l$)	0.038	17 (11-25) / 13 (9-19)

IQR, interquartile range

4.1.2 The biomarkers

4.1.2.1 Infections requiring antibiotic therapy

In the univariate analysis, elevated levels of WBC, neutrophils, CRP, PCT, IL-6, LBP and a reduced Hb were associated with an infection requiring antibiotics. In the multivariate analysis, only WBC, Hb and CRP remained as independent predictors.

In Sweden, both WBC and CRP are established acute markers routinely employed in the diagnosing of infections. They are, however, markers of a general inflammatory response and not specific to infections. Accordingly, the discriminatory ability was not overly impressive, with an AUC of 0.71 for CRP and 0.68 for WBC. The AUC difference between CRP and WBC was remarkably small, considering the unparalleled confidence the clinicians seemed to confer on CRP for antibiotic prescriptions; the decision to actually prescribe antibiotics was significantly associated with the CRP result, which was not the case for WBC and Hb. Furthermore, the accuracy of the clinician's antibiotic decision within the first 4 hours was not superior to the accuracy of CRP in identifying infections requiring antibiotics. All the other biomarkers, however, performed worse, and in anticipation of better markers CRP seems to deserve its position as the foremost guide for antibiotic decisions in the emergency care environment. It should be kept in mind, though, that the limited discriminatory ability leads to substantial overlap between patient groups (Fig. 9), making judgments based on individual results uncertain.

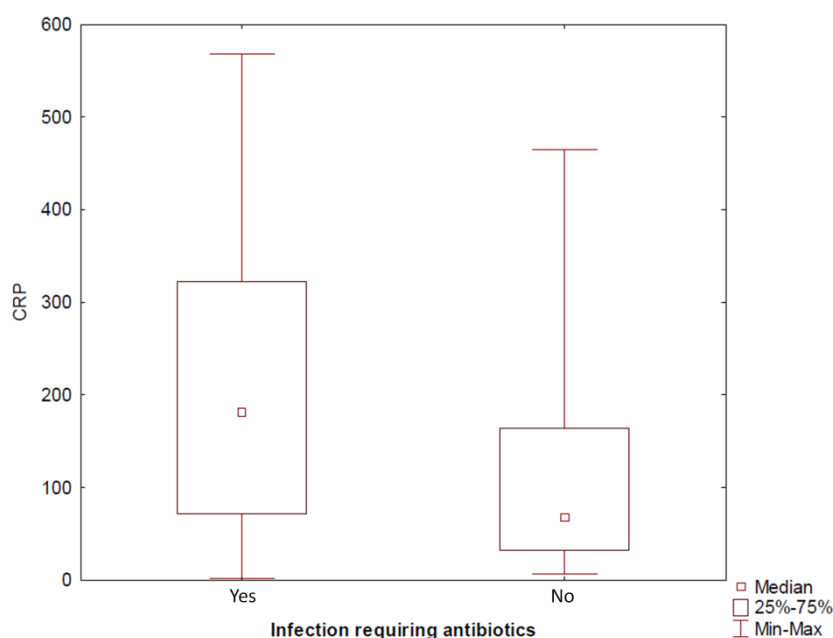


FIGURE 9. Box-plot representing the distribution of CRP results between patients with/without antibiotic need

Anemia is a common sign of chronic infections and inflammatory conditions [115], but a reduced Hb is not generally considered a marker of acute infection. Rather, sepsis patients are often hypovolemic on presentation with an expected increase in hematocrit and Hb concentration. Contradicting this view, our results suggest that a decreased Hb is a significant sign of an acute bacterial infection. This observation is biochemically explained by the rapidly altered iron metabolism induced by the inflammatory response. IL-6 and LPS trigger the hepatic release of the acute phase protein hepcidin, which inhibits duodenal absorption of iron. In addition, uptake of transferrin-bound iron into monocytes/macrophages is stimulated. Within hours, this process leads to a limited availability of circulating iron, resulting in a functional iron deficiency. The evolutionary background to this phenomenon is probably to be found in the microbes' indispensable need for iron as a substrate [33, 48, 115].

A number of the investigated biomarkers share an association with the hepatic acute phase response; IL-6 as an inducer, CRP and LBP as actual acute phase proteins, and Hb as a downstream response protein. Individually, they signaled significant alterations in response to infections requiring antibiotics, mirroring the importance of the acute phase response as a defense mechanism. Nonetheless, of these proteins only CRP and Hb retained an independent ability to predict this condition, Hb with the more modest AUC of 0.6, making individual interpretation even more difficult than for CRP and WBC.

PCT has been proposed as a more reliable marker of bacterial infection than CRP and other traditional tests [116], and has become increasingly introduced across acute care settings in recent years. Interestingly, in the multivariate analysis PCT failed to meet the expectations of a significant independent predictor of infections requiring antibiotics. There are some results from similar patient cohorts which support this finding [117, 118].

Taken together, we found that an introduction of biomarkers like PCT, LBP and IL-6 in the ED would not contribute significant guidance to antibiotic decisions. Furthermore, CRP, WBC and Hb provide valuable, but insufficient information for these critical judgments.

4.1.2.2 Bacteremia

“As regards prognosis, it is evident that a negative [blood] culture does not give much assistance, while a positive result gives a very unfavorable prognosis in the majority of cases” [46]. More than a century of medical development has passed since this assertion was published, and it still holds true; bacteremia is consistently a sign of severe infection [83, 119-121]. Bacteremic patients constituted a subset to the group of subjects with infections requiring antibiotics, and we wished to examine whether there were specific markers characterizing this population at risk of critical disease.

Of the biomarkers, an augmented WBC, neutrophil count, CRP, PCT, IL-6, LBP, lactate, D-dimer, urea, bilirubin, and a reduced platelet count was significantly associated with bacteremia on presentation. In the multivariate analysis, PCT, CRP and bilirubin remained independent predictors. For this condition PCT demonstrated a superior diagnostic accuracy; with the AUC of 0.77 it was the best total performance of any marker.

4.1.2.3 Severe sepsis

When analyzing the association between the studied variables and the presence of severe sepsis within the first 24 h of hospitalization, we excluded variables directly or indirectly instrumental in the definition of severe sepsis. Of the remaining variables (Hb, CRP, PCT, IL-6 and LBP), only IL-6 and PCT were significantly associated with severe sepsis. Thus, regarding discrimination between severe sepsis and other conditions in ED patients with a suspicion of severe infection, CRP appears not to be a valuable marker in this context.

4.1.2.4 Procalcitonin – a severity marker?

For this ED population, PCT did not provide valuable guidance on antibiotic decisions, but was significantly associated with bacteremia and severe sepsis, both potentially critical conditions. CRP did not predict severe sepsis and performed worse than PCT for bacteremia.

In an *a posteriori* analysis of the PCT distribution between these conditions, a picture emerged where PCT primarily seemed to respond to bacteremia. Indeed, the least severely ill bacteremic patients, those *without* severe sepsis, exhibited significantly higher PCT values than non-bacteremic patients *with* severe sepsis ($p=0.03$, data not shown). In fact, of patients without bacteremia only the most severely septic, those with multiple organ failure, demonstrated PCT results comparable with bacteremic patients without severe sepsis (Fig. 10). Within the bacteremic group, increments of sepsis severity were associated with increased PCT results. For non-bacteremic patients, PCT could not discern between patients without severe sepsis and those with severe sepsis and only one organ dysfunction. Thus it would seem as if PCT chiefly is a marker of the systemic inflammatory response seen in association with bacteremia. This is in line with the results of other authors, who also discuss the usage of PCT to exclude bacteremia and limit the number of drawn blood cultures [122-124]. Such a measure would have been injudicious in our cohort, as there was a small number of bacteremic patients with normal PCT results. Rather, the additional value of a single PCT measurement on ED presentation can be questioned, as there is ample access to alternative severity markers, e.g. those defining severe sepsis. Certainly, this does not preclude the potential success of repeated PCT measurements for monitoring purposes and for guidance on antibiotic duration, areas which have seen some promising results [125-128].

4.1.2.5 Notes on biomarkers

Although we could demonstrate a certain clinical utility for several of the investigated markers, it was limited. The ideal sepsis biomarker will provide information on the presence or absence of an underlying infection, the nature of the infectious insult (viral/bacterial, local/systemic etc.), severity of disease, prognosis, therapeutic alternatives and treatment response. Biomarkers exhibiting similar qualities have been introduced for other severe diagnoses, e.g. acute myocardial infarction. However, unlike distinct organ related conditions with a well characterized pathology, sepsis is more of a concept aiming to describe a clinically relevant syndrome. In sepsis,

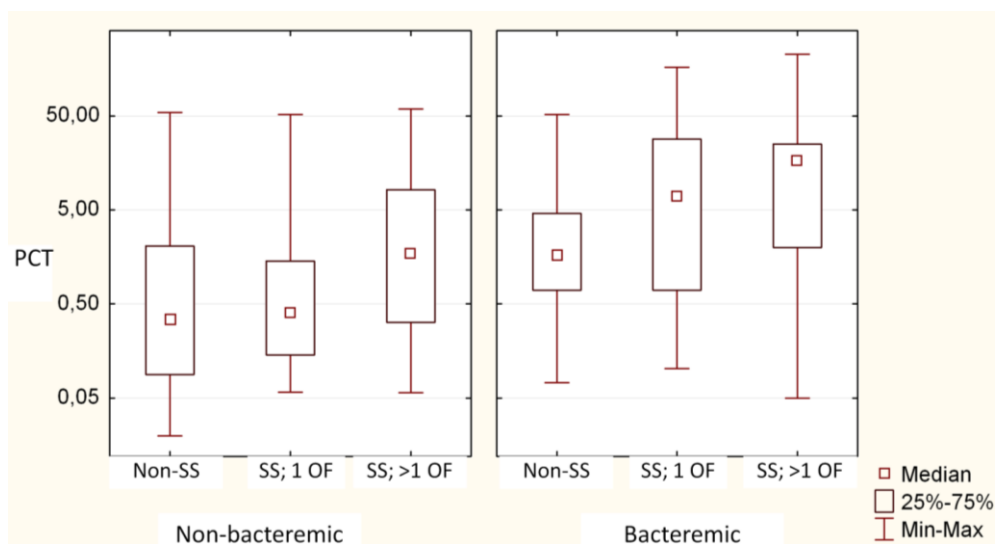


FIGURE 10. Box-plot representing the distribution of PCT results between patients with/without bacteremia. SS, severe sepsis; OF, organ failure

a variety of inherently different pathophysiological processes share the capacity to converge in a common clinical phenotype. In addition, the innate immune system reacts similarly in response to microbial structures (PAMPs) and endogenous alarmins (DAMPs), and establishing markers that reliably distinguish sepsis from SIRS of other origin has consequently proven to be a challenge. In summary, it seems unlikely that any single biomarker will become *the* universal sepsis marker. For the present, we will have to accept markers of a more narrow capacity, adapted to specific situations or conditions.

In emergency care settings it is of particular importance to distinguish patients at risk of imminent progression from those with a more limited disease. Hence, markers with an ability to mirror pathophysiological processes responsible for clinical deterioration have been of special interest, and for this reason a multitude of substances related to vasodilation, endothelial damage, and coagulation dysfunction have been tested [85]. So far, success has been limited, but there have been some promising reports. A recently discovered molecule associated with monocyte activation, sTREM-1, is upregulated in the presence of bacteria and fungi, and has been suggested as an important inducer of shock [129]. In clinical studies it has also been reported to perform better than CRP and PCT, both for the detection of infections and as a prognostic marker [130-132]. However, other authors have reported contradicting results in critically ill patients [133], as well as in ED patients with a suspected infection [134]. A recent meta-analysis which investigated the predictive ability for bacterial infections showed a pooled AUC of 0.91 for sTREM-1, indicating a high discriminative capacity [135]. This report did not address the prognostic ability, however.

Another biomarker which has attracted recent attention is HBP, a vasoactive mediator released from activated neutrophils. HBP was shown to be highly predictive of severe sepsis in a cohort of ED patients similar to the one we studied. With an AUC of 0.95, a remarkable performance for a sepsis marker, HBP could also predict circulatory failure in 29 of 32 patients hours before the onset of hypotension [86]. In subsequent studies of intensive care patients, however, these extraordinary results have

not been reproduced, in particular due to a rather poor specificity; it seems as if an increase in HBP levels is a more common finding in critically ill patients, regardless of infectious status [136, 137]. In addition, in a study of patients with shock, HBP could not distinguish between septic and non-septic shock [138]. Therefore, HBP may be a more general marker of critical disease. Nonetheless, for a population of ED patients with a high pre-test probability of severe infection, HBP appears to be a promising candidate marker for severe sepsis, and further validation from larger studies is desirable.

In recent years, several groups have also employed a strategy where information from biomarkers representing various aspects of the inflammatory response is combined to a more complete picture. With data from plasma concentrations of PCT, neutrophil expression of CD64, and sTREM-1, Gibot et al. created a score which outperformed all of the individual biomarkers when it came to diagnosing sepsis in critically ill patients [139]. For ED patients with suspected sepsis, Shapiro et al. used a biomarker panel of neutrophil gelatinase-associated lipocalin (NGAL), IL-1ra, and Protein C which was predictive of severe sepsis, septic shock, and death [140]. A Danish group evaluated a combination of six markers (CRP, PCT, neutrophils, sTREM-1, suPAR and MIF) for the detection of sepsis in a cohort of ED patients, and found the combination to be superior to the individual markers [134]. Thus, the application of composite markers holds some promise for improved diagnostic accuracy, and recent technological progress will provide ample opportunities for future studies within this field.

4.1.3 SIRS and severe sepsis

A closer examination of the prevalence and impact of severe sepsis and SIRS within the study population was performed and reported in paper II.

To make the relationship between SIRS and severe sepsis evaluable, SIRS was not considered a prerequisite for the diagnosis of severe sepsis, which is in accordance with current Swedish national sepsis guidelines [141]. Furthermore, we refrained from using the diagnosis septic shock, defined as persistent hypotension despite adequate volume resuscitation, since the definition is vague, subject to interpretation, and difficult to manage outside of the ICU in an environment where continuous monitoring of physiological parameters is not always feasible. Hence, all patients with hypotension induced by infection were classified as having severe sepsis.

4.1.3.1 SIRS

Not surprisingly, SIRS (Box 1) was a common finding within this cohort. Of patients with at least three registered SIRS parameters at ED presentation, 72% met the definition. Between 47% and 65% of the individual criteria were satisfied on presentation, the lower rate for RR and the higher for temperature (Fig 11). The presence of SIRS on arrival was significantly associated with a final diagnosis of an infection requiring antibiotics, but neither with severe sepsis nor severe sepsis with a critical course. Interestingly, we found that fulfilment of two of the individual

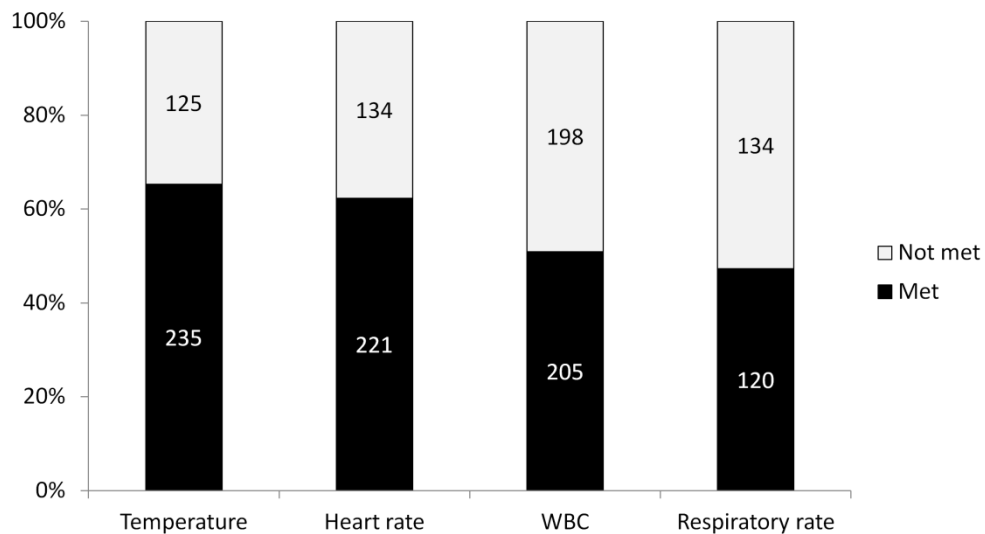


FIGURE 11. SIRS criteria met by patients on ED presentation, fractions. Figures within bars refer to the number of patients represented by the bar portion

criteria, WBC and RR, actually did correlate significantly with a diagnosis of severe sepsis within 24 h, in contrast to the temperature and heart rate criteria. An explanation for this could be that fever is a cardinal sign of most acute infections, regardless of severity, and thus highly unspecific. In an ED population based on the assumption of an infection, a high prevalence of fever is certainly to be expected. Moreover, since temperature and heart rate are covariates, patients with a high fever are likely to also experience tachycardia, and thus they will, conditionally, meet a second SIRS criterion. Hence, the inclusion of interdependent variables in a criterion based diagnosis must be regarded as fallacious; such variables should not receive the same weight as independent variables, or they will contribute to spurious results. In the case of SIRS, this is likely to result in an overly high sensitivity. In our study, a majority of the patients satisfied the SIRS definition regardless of the presence of infection, severe sepsis or bacteremia. Of patients with severe sepsis and a critical course, this fraction was as large as 88%. This result, based on a limited number of patients in our study, agrees with data from large ICU studies [28, 142]. To improve specificity, a stricter SIRS definition has been applied in some large studies, with a requisite of three satisfied criteria instead of two [143, 144]. When using this definition, we obtained a significant association between SIRS and severe sepsis, as well as between SIRS and severe sepsis with a critical course. Clearly, this involved an increased restrictiveness. Even among the critically ill subjects, 24% did not meet this stricter definition, and findings from larger series suggest that a large proportion of critically ill infected patients will be excluded by a stricter definition [142, 145]. If used in clinical trials, this definition will involve a selection bias that will limit the external validity of the results.

In summary, our results suggest that SIRS status defined on arrival in the ED is a both non-discriminatory and potentially restrictive tool of poor value for the identification of severe sepsis and prediction of a sepsis associated critical course, a view which has been substantiated by similar observations in studies of various

populations [28, 106, 145-148]. It is time for the classical SIRS definition to be abolished.

4.1.3.2 Severe sepsis, organ dysfunction and critical course

There were 110 patients (29.7%) who met the criteria for severe sepsis on ED presentation, and after the first 24 h of hospitalization there were in total 156 patients (42.1%) who had developed an organ dysfunction, making severe sepsis a common condition within this cohort. In the vast majority of cases, the cause was bacterial ($n=139$; 89.1%). Out of seven possible dysfunctions defining severe sepsis (hypotension, hypoperfusion, respiratory, neurologic, renal, coagulatory and hepatic), no patient had more than four, and a single organ dysfunction was most abundantly observed ($n= 90$; 57.7%). Only 20 (12.8%) of the patients with severe sepsis sustained a critical course, defined as either death within 28 days ($n=8$; 5.1%) or subsection to intensive care ($n=14$; 9.0%). The relations between the number and type of organ dysfunctions are illustrated in figure 12 and 13.

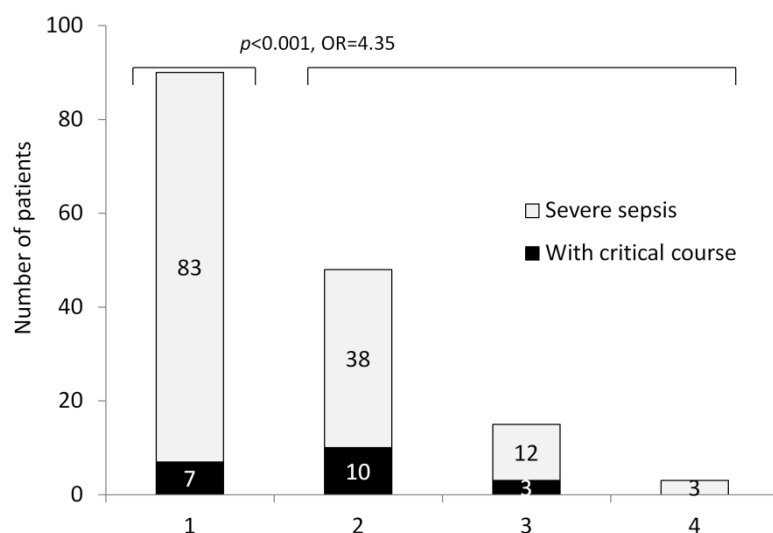


FIGURE 12. Number of organ dysfunctions observed in patients with severe sepsis and in those who developed a critical course. Figures within bars refer to the number of patients represented by the bar portion. Having more than one organ dysfunction was significantly associated with a critical course. OR, odds ratio.

Our results suggest that severe sepsis is a common occurrence in patients admitted with suspected infections during the first 24 h of hospital stay. The most striking observation, however, was the low case-fatality rate (5.1%; 95% CI 1.7-8.6%). Traditionally, this figure has been reported to be several times higher for severe sepsis, often around 30% or more [3, 8, 10, 144]. This raises some questions with regard to heterogeneity between studies. Was this a treatment effect? Or, was our study population inherently different from most other populations? If so, how? Was there a classification bias?

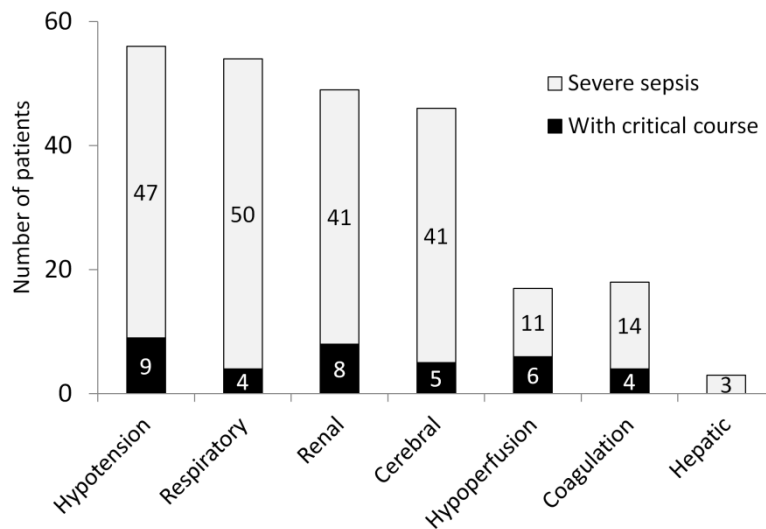


FIGURE 13. Types of organ dysfunctions observed in patients with severe sepsis and in those who developed a critical course. Figures within bars refer to the number of patients represented by the bar portion.

When it comes to such a multifaceted syndrome as sepsis, issues like these can be dwelt upon at length. For the sake of brevity, this discussion will be restricted to a few aspects.

First, our patients were attended to by infectious diseases physicians, especially trained to detect and treat these conditions, which is not the case in most ED settings. Did this lead to earlier detection and superior treatment? There may be unrecognized data supporting this, but when it came to one important factor, time to antibiotics, this was not the case. The median time to antibiotic therapy was 2.25 h from presentation, not better than what has been reported in ED studies with higher mortality [149, 150].

Second, this was a study addressing an ED population, while most sepsis studies historically have been performed in ICU settings, where mortality rates generally are higher than in other hospital contexts. Nevertheless, one of the *raison d'être* for the present sepsis definitions was improved generalizability between studies [23]. If the occurrence of intensive care is an inherently better marker of severity than what can be inferred from the sepsis definitions *per se*, then we must ask ourselves to what extent they are purposeful. It may be that the severe sepsis criteria better define a population at risk of, rather than established critical disease, which is the case for ICU patients.

The definitions of severe sepsis are dichotomous; any of a number of organ failures will satisfy the diagnostic criteria. Furthermore, once an organ dysfunction criterion is met, subsequent grading of the dysfunction is not supported. Within the field of intensive care, this issue has been negotiated by the establishment of severity scoring systems, e.g. the SOFA score, once introduced for the assessment of sepsis induced organ failures [151]. The SOFA score has demonstrated a fairly good accuracy of mortality prediction also in the ED, and has been suggested as an additional tool for risk stratification and prognostication of ED patients with severe sepsis [152]. While we did not apply the SOFA score in this study, it may be worth considering for future studies.

Third, the greater part of severe sepsis patients in our cohort only had one organ dysfunction, of whom only 8% suffered a critical course. On the other hand, the most common dysfunction was hypotension, a marker of global systemic failure associated with poor outcome [153]. Most of our patients, however, responded to fluids and hypotension was a transient phenomenon. Also, the presence of hypotension was not significantly predictive of a critical course when compared to other organ dysfunctions.

While hypotension can be described as a marker of macrocirculatory failure, hypoperfusion, as represented by hyperlactatemia, is considered a marker of deranged microcirculation resulting in tissue hypoxia. In the last decade, lactate has emerged as a preeminent prognostic marker among the sepsis defining criteria [11, 78, 153-155]. There were relatively few of the patients in our study who presented with hyperlactatemia. Of those, however, 35% sustained a critical course. This proportion was higher than for other organ dysfunctions, albeit not significantly. It cannot be excluded though, that the low overall incidence of hypoperfusion was representative of a less critically ill population.

Finally, the devil is in the details, and when it comes to sepsis, the definitions. In reviewing a substantial number of previously published studies for their respective definitions of severe sepsis, a great variety among the definitions used, pertaining to both SIRS and the various organ dysfunctions, was noted. Selected examples

TABLE 3. Examples of organ dysfunction definitions from various sepsis studies.

Hypotension	Hypoperfusion	Respiration	CNS	Renal	Coagulation
SBP<90 or MAP<70 ≥1h despite fluids	pH ≤7.30 or BE≥-5 and Lactate>1,5x ref.max	PaO ₂ /FiO ₂ <280(mmHg)	Acute altered mental status (GCS<14)	<0,5ml/kg/h for 1h	Platelets low or ↓>25% + ↑INR+↑APT or bleeding
SBP<90 >1h despite fluids	Lactate>2 mmol/l	PaO ₂ <70mmHg (air) PaO ₂ /FiO ₂ <280(mmHg)	Acute altered mental status	Creatinine ≥300 μmol/l or < 500 ml/d	Platelets<100
SBP<90 or ↓40	pH ≤7.30 or Lactate>2,5	RF>24 or ventilator	GCS≤11	<0,5ml/kg/h for ≥2h despite fluids	Platelets<100or ↓>50% or INR>2 or ↑APT≥20%
SBP<90 or ↓40 >1h despite fluids	Lactate> ref.max or BE≥-5 or pH<7,3	PaO ₂ ≤75mmHg (air) PaO ₂ /FiO ₂ ≤250(mmHg)	↓GCS≥2p	<30 ml/h for ≥1h	2 of: A. Platelets<75 or ↓>50% B. INR>1,5 C. D-dimer >0.5orFDP>10
MAP<70 ≥0,5h despite fluids	Lactate>2 mmol/l or BE>-5	PaO ₂ /FiO ₂ <300(mmHg)	GCS≤9	<0,5ml/kg/h for 2h despite fluids or Creatinine ↑>45 μmol/l	Platelets<150

SBP, systolic blood pressure; MAP, mean arterial blood pressure; BE, base excess; PaO₂, partial arterial oxygen pressure; FiO₂, fraction of inspired oxygen; RF, respiratory frequency; GCS, Glasgow coma scale

are presented in table 3. All studies referred to either the 1992 or the 2001 consensus definitions, sometimes with the addition “modified from”, or a similar wording. Furthermore, numerous studies do not report their organ dysfunction criteria in detail. Thus minor variations within seemingly identical definitions could potentially constitute heterogeneous study populations, influencing observed frequencies of, e.g., severe sepsis and sepsis attributed mortality. Indeed, this was recently illuminated by a Dutch group that applied various definitions used in earlier landmark studies to their own ICU population. The range of observed incidences of severe sepsis varied substantially with the definitions used, between 6 and 27% [156]. To this should be added considerable diversity in the definitions of infection between studies, varying from microbiologically confirmed to clinically suspected with antibiotic treatment. Our definitions may have been at the milder end of the organ dysfunction spectrum, thus limiting the case fatality rate. In fact, one large sepsis ED study using similar definitions reported a severe sepsis mortality of 9.2% [106]. This was still nearly twice ours, although this difference could probably be attributed to dissimilar background populations, sheer random variation, or a combination thereof.

In my view, a set of definitions similar to the ones we used may well be appropriate for the discrimination of infected patients at risk of progression to a more critical stage of disease. As a whole, however, the field of clinical sepsis research is in urgent need of more precise and unambiguous definitions. In the 2001 revision of the consensus definition of sepsis the so-called PIRO concept was launched [29]. This theory recognized the shortcomings of the original definition, and proposed a novel multi-dimensional approach, seeking to characterize predisposing (P) factors like comorbidity and genetic polymorphisms; the insult (I), i.e. the nature of the infectious source; the pathophysiological and immunological response (R); and the extent of organ dysfunctions (O). After more than a decade, the PIRO model remains a beautiful hypothesis in search of practical applications, and it will take tremendous efforts to develop it into a useful and validated tool for research and clinical practice. Yet, in recent years we have seen unprecedented advances in areas like gene expression profiling, proteomics, nanotechnology, and techniques for microbial detection. Through these methods we may gain a more sophisticated understanding of underlying mechanisms, hopefully sufficient for the construction of more accurate definitions and interventions for the clinical phenotype we refer to as sepsis.

4.2 PAPER III

Cytokines are fundamental to the coordination of the inflammatory response. During sepsis, these proteins abound in plasma and most pro- and anti-inflammatory cytokines have been associated with a critical course [94]. Still, no cytokine has stood the test of a clinically relevant marker, and the role and origin of circulating cytokines remain incompletely elucidated. We hoped to clarify whether blood dwelling leukocytes express cytokines during sepsis by using the sensitive ELISpot technique, which measures the number of actively cytokine secreting cells instead of the circulating amount of cytokines. We also aimed to explore whether the secretion pattern would provide a more accurate and clinically useful representation of the disease process. Blood from sepsis patients and healthy controls was sampled and transported to the laboratory in a timely fashion for the immediate initiation of the *ex vivo* ELISpot

analysis, while plasma was reserved for cytokine measurement by Luminex methodology.

4.2.1 Spontaneous cytokine secretion in sepsis and endotoxemia

Most patients were critically ill, 24 out of 32 (75%), suffered from septic shock, and 18 (56%) had bacteremia. In such critically ill patients, endotoxemia is a common phenomenon [157], and it was reasonable to anticipate high levels of circulating PAMPs in this cohort. Somewhat to our surprise, the resulting numbers of spontaneously cytokine secreting cells were very low overall, with the largest number seen for TNF-secreting cells. There were, however, no significant differences in the number of secreting cells between patients and controls for any of the investigated cytokines. This lack of *in vivo* activated, cytokine secreting cells in the circulation was evident even in patients with ongoing bacteremia, and irrespective of the interval between the first signs or symptoms of sepsis and blood sampling (9-337 h). In contrast, plasma levels of the examined cytokines were elevated in sepsis patients, as expected (Fig. 14).

Since the so-called “cytokine storm” in sepsis is believed to be an early phenomenon, we hypothesized that the cells might have been exhausted or in a compensatory dormant state at the time of blood sampling. For this reason we employed a model of human endotoxemia to trigger a “septic” state in healthy volunteers, enabling us to study the earliest phase of cytokine secretion after TLR activation. In blood collected before and at 30 and 150 min after LPS injection, results were consistent with the previous results; there were few or no cytokine secreting cells. On the other hand, we found plasma concentrations of TNF and IL-6 to be substantially elevated at 150 min. At the same time we observed a notable monocytopenia, characterized by a 95% reduction from baseline.

In summary, we can conclude that regardless of bacteremia, endotoxemia, sepsis stage or presence of circulating cytokines, there were few or no actively secreting cells in samples from the bloodstream. Our findings suggest that the origin of plasma cytokines in sepsis and endotoxemia is not to be found in the population of circulating leukocytes. The rapid reduction of monocyte counts seen after LPS injection is a familiar phenomenon [158] which probably represents a programmed egress by activated monocytes into tissue after an inflammatory stimulus [159]. Plasma cytokines most likely are produced by such extravasated monocytes, or possibly by resident tissue macrophages. This is an interesting finding since models for *ex vivo* and *in vitro* analyses of blood cells are frequently used for the exploration of intra- and intercellular pathways of the innate immune response. Our results suggest that interpretations regarding the cytokine kinetics seen in sepsis should be made with caution when based on data from studies of circulating leukocytes, as these cells do not appear to contribute to this process.

An alternative explanation to the negative results could be that the collecting of blood and subsequent handling of cells for the *ex vivo* analysis could have “shut down” the sampled cells, leading to false negative results. There are some data that contradict this hypothesis. First, in a supplementary experiment we exposed whole blood from healthy volunteers to comparable LPS concentrations *ex vivo*, after which cell separation and ELISpot analysis detected cytokine secreting cells of a contrastingly high frequency. Second, once the cellular process of transcription, synthesis and

secretion of cytokines has been instigated, mechanisms that reverse this process seem to be equally complex, primarily aiming at transcriptional inhibition, a time consuming process [160]. Third, as discussed below, we applied a “second hit” model, in which the cells were exposed to endotoxin *ex vivo*, which led to significantly increased numbers of cytokine secreting cells from both patients and volunteers, thus exhibiting a sustained secretory capacity (Fig. 15).

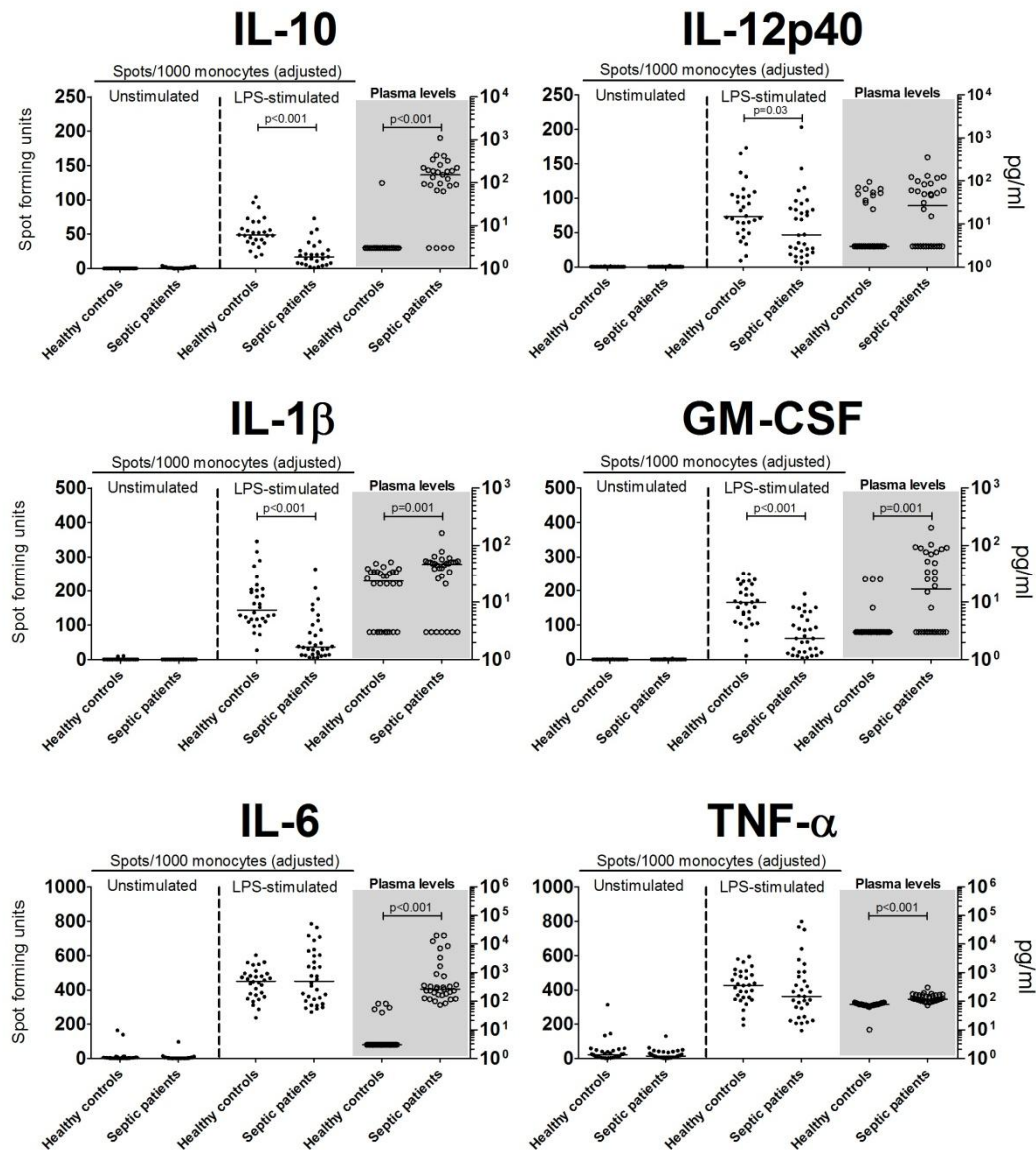


FIGURE 14. ELISpot analysis and plasma cytokine concentrations of IL6, TNF- α , IL-1 β , GM-CSF, IL-10 and IL-12p40 in sepsis patients and healthy controls. Each graph displays spontaneous and LPS-induced cytokine secretion expressed as spots/1000 monocytes and plasma concentrations as pg/ml. Data is shown as individual values and horizontal bars indicate the median value for each group.

4.2.2 Cytokine secretion after *ex vivo* LPS stimulation

After exposure to endotoxin, a notable proliferation of cytokine secreting cells was observed in samples from both patients and controls. Interestingly, the cells from sepsis patients showed a selective down-regulation of GM-CSF, IL-1 β , IL-10 and IL-12p40 after *ex vivo* LPS stimulation in comparison with the healthy controls. This was not the case for TNF and IL-6 producing cells, where similar numbers were observed between the groups.

Subsequent to an inflammatory response, whether induced by infection, trauma or experimentally, a resulting hypo-responsiveness of immune cells or organisms to further immune stimulation is commonly observed. This phenomenon, often referred to as “endotoxin tolerance”, is believed to be a protective measure by which the organism balances the deleterious consequences of a protracted inflammatory response. Accordingly, monocytes from sepsis patients have been shown to express reduced amounts of a vast array of cytokines in response to endotoxin, among them TNF and IL-6 [70, 71, 73, 161]. This appeared contradictory to our findings, where the TNF and IL-6 secreting capacity of monocytes from sepsis patients was preserved, in contrast to the other investigated cytokines. Thus, we concluded that the downregulation of cytokine secretion appears to be selective, sparing TNF and IL-6, at least with respect to the number of secreting cells. This was an unexpected finding, and the cause of this observation remains undisclosed. Nevertheless, due to methodological differences our results were not necessarily incompatible with earlier reports of a diminished production of these cytokines. While ELISpot is a very sensitive method for the detection of secreting cells, it does not quantify the amounts of secreted cytokines. For this reason the number of cytokine secreting cells does not necessarily correlate with the amounts of cytokine secreted. Also, it should be emphasized that ELISpot detection of secreted cytokines is inherently different from “upstream” measurement of intracellular synthesis by methods like gene-expression and intracellular staining, which may lead to divergent results.

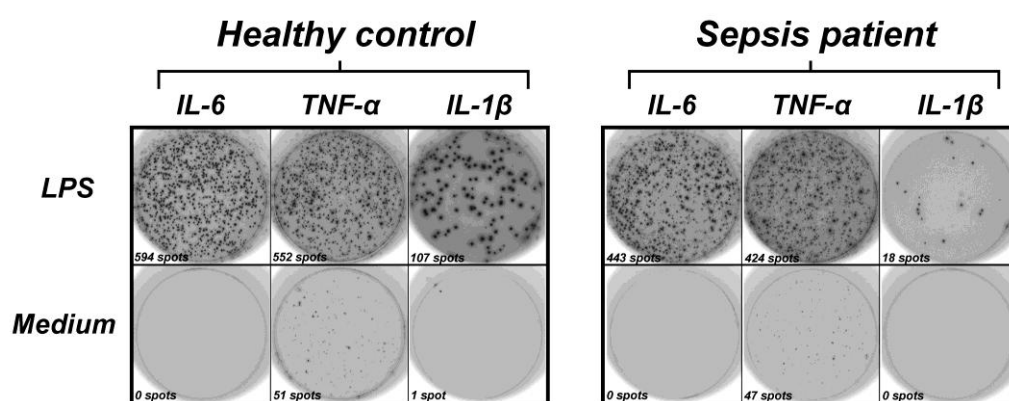


FIGURE 15. Examples of IL-6, TNF- α and IL-1 β spots generated with leukocytes from one healthy control and one sepsis patient in the ELISpot assay. Cells were incubated in the presence of LPS (2 ng/ml) or cell culture medium alone.

4.2.3 Clinical correlation

As mentioned earlier, the evolution of intensive care during the last century has brought the successful treatment of previously lethal diseases within the range of possibility. Although there is undoubtedly a potentially life-threatening early pro-inflammatory phase of sepsis [63], mortality is today more common among patients who, with the help of supportive care, have survived this initial stage [43, 162]. In addition, it has been increasingly recognized that the modulating anti-inflammatory response (“CARS”) is not following upon the initial proinflammatory phase (“SIRS”) in a two-wave pattern; it is rather a parallel process where anti-inflammatory cytokines like IL-10 are detectable soon after an endotoxin challenge [158], as well as in early septic shock [163, 164]. It has recently been shown that also TNF, key instigator of the proinflammatory response, induces tolerance to endotoxin in macrophages [165].

Whether this prompt downregulation of the immune defence is mainly beneficial or deleterious is the topic of an ongoing scholarly discussion. On the one hand, there is evidence of a compartmentalization of the immune response, where the blood stream represents an immunosuppressive milieu with desensitized immune cells, while cells of other compartments display a retained or even increased reactivity [166, 167]. This is thought to favour containment of an infectious focus, while the systemic response is tuned to a less aggressive mode. On the other hand, sepsis-induced immunosuppression is believed to be responsible for an impaired clearance of microorganisms, reactivation of latent viruses and a reduced resistance to secondary opportunistic infections [168]. Indeed, a persistently reduced monocyte HLA-DR expression has been predictive of both mortality [169] and nosocomial infections in septic shock [170], while autopsy studies have shown that immune cell anergy is not confined to the blood compartment in sepsis [171]. Consequently, immunostimulatory therapies with the potential to reverse this suppressed state have become a hot topic, although with a more cautious approach in view of the disappointing experiences from previous immunomodulator trials [172]. The need for correct determination and stratification of patients’ immune status is now recognized, and functional testing of monocytes has been suggested as one of several tools suitable for this purpose [173]. For this reason we wanted to assess the capacity of cytokine secretion from sepsis patients with regard to disease severity and stage of disease. Whether measured by sepsis category (shock vs. non-shock), SOFA score or APACHE II score, we found no correlations with the plasma levels of cytokines. In contrast, we observed significantly fewer monocytes from patients with higher SOFA scores secreting GM-CSF, IL-1 β , IL-10 and IL-12p40 in response to LPS stimulation *ex vivo* (Fig. 16). Similar differences were found between patients with and without septic shock. With respect to TNF and IL-6 secretion, there were, as previously found, no significant differences. We concluded that the degree of sepsis-induced organ failure, as measured by the SOFA score, was indeed reflected in the degree of failure to secrete certain cytokines at the single cell level.

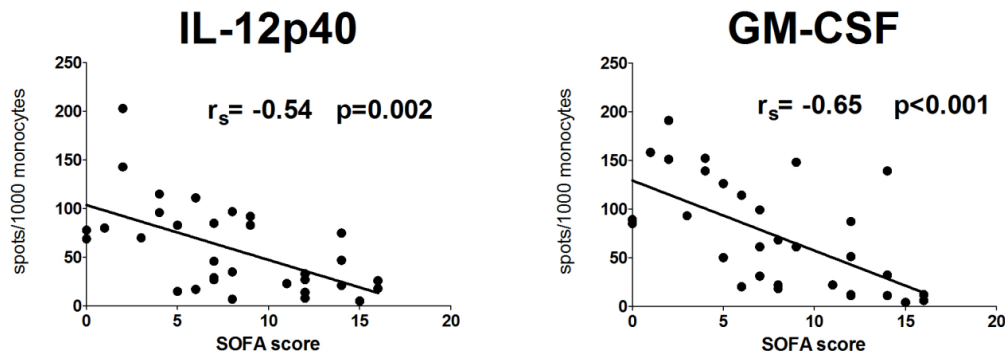


FIGURE 16. ELISpot results for IL-12p40 and GM-CSF demonstrating a significant negative correlation between LPS induced cytokine secretion and SOFA score. r_s , Spearman's rank correlation coefficient

Accurate assessment of the sepsis-associated Response is one of the cornerstones of the aforementioned PIRO-model. Grading of patients' current immune status is likely to become an important part of this. Due to the multifaceted nature of sepsis-induced immunosuppression [168], simultaneous gauging of several immune functions may be required, including tests of monocyte responsiveness. Our results indicate that measurement of the cytokine secreting capacity at the single-cell level may contribute favorably to this end, although further validation from larger-size studies is warranted. It is to be noted, however, that the value of this measure is largely dependent on whether abnormal findings are amenable to treatment, and further, whether this will ultimately improve patients' outcome. Otherwise, this will be just another severity marker of limited clinical utility.

4.3 PAPER IV

This was a preparatory study for the subsequent work on sepsis patients (paper III). It was designed to answer basic questions regarding the cytokine secreting repertoire of leukocytes participating in the innate immune response to infection.

We hypothesized that the ELISpot method, which detects individual cytokine secreting cells, would more accurately determine the cytokine profile of PMN and PBMC than the commonly used ELISA method, which measures the total amount of secreted cytokines in cell supernatants. The latter approach is more susceptible to the influence of occasional contaminating cells; addition of a few high producing cells, like monocytes, to a population of low producers, e.g. granulocytes, may skew the results. In contrast, with the ELISpot technique any cytokine producing cell will be represented by no more than one spot, thus minimizing the risk of significant influence from individual contaminating cells.

A panel of 8 cytokines (IL-1 β , IL-6, IL-8, IL-10, IL-12, TNF, GM-CSF and MIP-1 β) was tested. In addition, PMN secretion of IFN- γ , perforin and granzyme B was analyzed. Cells from healthy donors were cultured in medium alone or stimulated by LPS in parallel experiments.

4.3.1 Cytokine release from mononuclear cells

In PBMC preparations we found relatively high spontaneous secretion of IL-8, MIP-1 β , and (to a lesser extent) TNF in comparison with the other cytokines. Stimulation by LPS induced significantly increased frequencies of secreting cells for all the tested cytokines except IL-8, an important chemokine (chemotactic cytokine) predominantly responsible for the recruitment of neutrophils to the site of inflammation. Instead, larger and denser IL-8 spots were formed in response to LPS, plausibly representative of an augmented secretion from individual cells.

Monocytes are considered the main producers of cytokines among blood cells [174]. They constitute a minority of about 10-30% of PBMC, while the lymphocyte family forms the absolute majority. To also characterize the cytokine secreting ability of non-monocytes, we depleted the PBMC population of monocytes via magnetic CD-14 ligation, and repeated the former experimental procedure. For all the investigated cytokines, the secreting cell populations were reduced by 91-99% after LPS stimulation, confirming the role of monocytes as supreme cytokine producers.

4.3.2 Cytokine release from polymorphonuclear cells

Neutrophils are the frontline soldiers of the immune defense and the most prevalent of all leukocytes, normally constituting over 90% of PMN. They share their phagocytic capacity with monocytes and macrophages, but unlike these they are known to produce only modest amounts of cytokines. Nonetheless, human neutrophils have been reported to produce a wide variety of cytokines, some of which are surrounded by controversy [175, 176]. This is exemplified by a lack of consensus in the literature regarding the neutrophil ability to produce IL-6 and IL-10 [176]. Variations in the purity of cell preparations and the concomitant risk of monocyte contamination have been suggested as a source of conflicting results [176, 177]. When we explored the secreting capacity of isolated PMN, we found evidence of spontaneous secretion of the chemokines IL-8 and MIP-1 β , albeit to a lower degree than in PBMC populations. In response to LPS stimulation, the frequencies of IL-8 and MIP-1 β secreting cells increased significantly, while low numbers of TNF secreting cells also emerged. There were no cells found to secrete IL-10, IL-12, GM-CSF, IFN- γ , perforin or granzyme B. Furthermore, there were only occasional spots representing IL-6 and IL-1 β secreting cells. These spots appeared very similar to those produced by PBMC, suggesting monocyte contamination. A few similarly large and dense TNF spots, clearly distinct from the majority of weaker TNF spots produced by PMN, reinforced this impression. From these observations we inferred that the PMN cytokine repertoire is restricted to a few cytokines. This finding is supported by others using highly purified PMN separations [37, 178].

4.3.3 ELISA versus ELISpot in polymorphonuclear cells

The ELISpot technique was also compared to ELISA for the detection of TNF, IL-6 and IL-1 β in PMN. With regard to the number of cells needed to detect cytokine secretion, we found ELISpot to be more sensitive by a factor of at least ten. As a matter of interest, we found a discrepancy between the number of secreting cells detected by ELISpot and the amount of cytokines found by ELISA. Based on our previous findings, the observation of the highest number of spots for TNF was expected, as was the low

number of observed IL-6 and IL-1 β secreting cells. Conversely, the highest cytokine concentration detected by ELISA was found for IL-6, followed by IL-1 β and TNF. We suggest that this may be due partly to the contamination of occasional high-producing monocytes, partly to cytokine interaction with cell-bound and soluble receptors in supernatants after secretion.

In summary, we found ELISpot to be a useful tool for cytokine detection in various leukocyte populations. In addition, we confirmed that monocytes possess the main cytokine secreting capacity among blood cells and, finally, that the cytokine arsenal of PMN appears to be limited to a few cytokines.

5 MAIN FINDINGS

- For a selected population of ED patients with a high pre-test probability of a severe bacterial infection we concluded that an increased level of the routinely tested markers **CRP**, **WBC**, **RR** and a decreased **Hb** level contributed independently to an accurate selection of patients for antibiotic therapy.
- The more novel markers **PCT**, **IL-6** and **LBP** did not confer additional information for an antibiotic decision.
- The diagnostic accuracy was poor for all of the tested biomarkers. Consequently, there is a compelling need for better markers in order to facilitate antibiotic decisions and to strengthen antibiotic stewardship.
- **PCT** proved to be the best severity marker, independently associated with both bacteremia and, to a lesser extent, with severe sepsis.
- Severe sepsis was found to be of frequent occurrence among these patients (42%), while mortality was low (5%) in comparison to previous reports.
- The classical SIRS definition was met by a majority of the patients and did not contribute to the classification of sepsis. It should be abolished.
- By using the sensitive ELISpot technique we could conclude that circulating leukocytes are not the source of the elevated plasma levels of cytokines seen in sepsis.
- There was a selective sepsis-induced downregulation of the cytokine secretion in response to LPS stimulation *ex vivo*, reflected in decreased numbers of IL-1 β , IL-10, IL-12p40 and GM-CSF secreting cells, while the number of TNF and IL-6 secreting cells remained similar.
- The reduced number of cytokine secreting cells correlated with disease severity as measured by SOFA-score, underscoring the link between disease progression and immune status.
- In blood from healthy donors we confirmed that monocytes are the principal cytokine releasing cells, while neutrophil release was restricted to only a few cytokines.

6 FINAL REFLECTIONS

“When you know a thing, to hold that you know it; and when you do not know a thing, to allow that you do not know it - this is knowledge.”

- CONFUCIUS

More than anything, this work has taught me how challenging and elusive the field of sepsis is. To my mind, more far-reaching and comprehensive conclusions are difficult to draw from our findings. However, when looking at the individual pieces, there are a few points I would like to make which may be of some relevance.

Our observations suggest that, for ED patients with a suspected infection, the traditionally used biomarker CRP provides a better basis for an accurate antibiotic decision than the highly marketed PCT. The legitimate demand for better biomarkers, coupled with the constant discoveries of new candidate molecules and commercial interests, may result in an exaggerated enthusiasm. Therefore, it is important that the introduction of new biomarkers for predefined patient populations is preceded by appropriate validation studies. In addition, the purpose of the test should be well defined. Although a truism, this has not always been the case.

It seems obvious that the present sepsis definitions, designed to cure heterogeneity and poor generalizability between studies, do not fulfill their purpose. It has been repeatedly shown that the 1992 SIRS definition lacks discriminative ability, now also by us. Yet, it is still widely used as an inclusion criterion, probably for one reason only: everybody else does the same thing. This is not a valid argument. In addition, diffusely defined organ dysfunction criteria make the sepsis definitions subject to interpretation, leading to variations between studies which hamper the external validity. More detailed definitions and standardized inclusion criteria are required to improve our chances of developing better therapies and biomarkers.

Even with more strict definitions, the multifaceted clinical phenotype of sepsis is the result of various underlying pathophysiological mechanisms. To enable specific, biologically tailored interventions, these processes need to be properly distinguished. As a part of this quest, we chose to explore immune cell signaling by employing the ELISpot method. We found that circulating leukocytes were not the source of the increased levels of plasma cytokines. We also showed that the sepsis-induced downregulation of the cytokine secreting ability appeared to be restricted to some cytokines but not to others. These findings point to some topics apt for future investigations. While circulating blood cells are easily accessible, they may not be as relevant to the disease process as cells from other compartments. Efforts should be made to enable the study of cells from other locales in sepsis, in particular from infectious foci. Furthermore, by simultaneously applying several methods, e.g. flow cytometry and mRNA quantification in addition to ELISpot, a more complete picture of the subtle mechanisms that regulate cell signaling in sepsis may emerge. As this knowledge accumulates, we will eventually be able to delineate and address the distinct

pathological processes which share the capacity to induce sepsis. This will allow the current sepsis definitions to be disassembled and replaced by better validated and more relevant disease descriptions.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

7.1 BAKGRUND

Sepsis, i dagligt tal blodförgiftning, är en allvarlig sjukdom med ett ofta fortskridande förlopp. Sjukdomen klassificeras vanligen utifrån tre stadier av ökande svårighetsgrad, "sepsis", "svår sepsis" och "septisk chock". De svårare formerna anses orsakade av ett överdrivet kraftigt immunsvaret vid svåra infektioner. Detta leder till en frisättning i blodet av en mängd ämnen, bl a en grupp signalproteiner som kallas cytokiner, som gemensamt orsakar kärlläckage, lågt blodtryck och organsvikt. Paradoxalt nog anses denna överreaktion snabbt kunna förbytas i en obalans åt andra hållet, med ett skadligt försvagat immunförsvar som resultat.

Antalet fall av sepsis i västvärlden (Europa och USA) kan, lågt räknat, beräknas till mer än 2 miljoner fall årligen av vilka en tredjedel har svår sepsis eller septisk chock. Dödligheten inom denna tredjedel varierar mellan olika studier, men är fortsatt hög, c:a 25-50%. Det är klart visat att tidig behandling i sepsisförloppet påverkar utgången. En snabb upptäckt av tillståndet är därför av yttersta vikt. Resultaten från bakterieodlingar kommer inte förrän efter flera dagar medan vanligt förekommande blodanalyser visserligen uppfyller krav på snabbhet men inte är konklusiva och främst tjänstgör som stöd i den kliniska bedömningen. Samtidigt bidrar onödig antibiotikabehandling till den ökande globala resistensutvecklingen bland sjukdomsalstrande bakterier. Det finns således ett stort behov av ny diagnostik för att på ett snabbt och säkrare sätt identifiera såväl de patienter som är på väg in i ett livshotande tillstånd, som de som inte har antibiotikakrävande infektioner.

7.2 PROJEKTBEKRIVNING

- A. *Kritisk granskning av det diagnostiska värdet av ett antal kliniska och kemiska markörer i en grupp patienter med hög risk att utveckla svår sepsis.*

Sammanlagt undersöktes 404 patienter som lades in på infektionskliniken från Karolinska universitetssjukhusets akutmottagning med en misstänkt allvarlig infektion. Resultaten redovisades i delarbete I och II.

I det första arbetet utvärderades förmågan hos ett antal mätningar av basala fysiologiska kroppsfunktioner och blodprover att identifiera antibiotikakrävande infektioner, bakterieväxt i blodet och svår sepsis.

Sammanfattningsvis konstaterades att ett antal väletablerade prover (C-reaktivt protein, antal vita blodkroppar, hemoglobinhalt) och mätning av andningsfrekvensen kan hjälpa läkaren till ett korrekt antibiotikabeslut, men att tillförlitligheten hos dessa prover är otillräcklig. Samtidigt kunde nyare inflammatoriska prover (procalcitonin, interleukin-6 och lipopolysackarid-bindande protein) inte bidra till denna diagnos. Dock var procalcitonin överlägset C-reaktivt protein i diagnostiken av de mer allvarliga tillstånden svår sepsis och bakterieväxt i blodet.

I det andra arbetet kartlades förekomsten av svår sepsis hos patienterna och dess påverkan av det kliniska utfallet (vårdnivå och dödlighet inom 28 dygn). De variabler som definierar det s k system-inflammatoriska svarssyndromet (SIRS), som anses

föregå en fortskridande sepsissjukdom, analyserades också i relation till det kliniska förloppet. Dessa variabler utgörs i korthet av onormal temperatur, förhöjd puls, förhöjd andningsfrekvens, samt en onormal nivå av vita blodkroppar. Två av dessa fyra kriterier skall uppfyllas för att kunna ställa diagnosen SIRS.

Av patienterna uppfyllde 30% kriterier för svår sepsis redan på akutmottagningen. Efter 24h hade denna andel ökat till 42%. Endast en mindre andel patienter med svår sepsis (13%) utvecklade ett kritiskt förlopp, definierat som intensivvårdsbehov eller död inom 28 dygn. Dödligheten var endast 5%, en internationellt sett låg siffra.

Vid ankomst till akutmottagningen uppfyllde 72% av patienterna definitionen för SIRS. Någon betydande skillnad avseende förekomst av SIRS återfanns inte mellan patienter som hade/utvecklade svår sepsis och övriga, inte heller mellan patienter med svår sepsis med ett kritiskt förlopp och övriga.

Vi konstaterar att svår sepsis är ett vanligt tillstånd inom det första vård dygnet hos patienter som läggs in med misstanke om en allvarlig infektion, men att både intensivvårdsbehovet och dödligheten är relativt låga. Vidare att SIRS-diagnosen är ett dåligt instrument för att urskilja och förutsäga svår sepsis.

B. Introduktion och utvärdering av ELISpot-tekniken inom sepsisområdet för att öka kunskapen om cytokinproduktionen på cellnivå vid sepsis.

ELISpot är en känslig metod för att upptäcka cytokinfrisättning från immunceller. Vi hoppades att genom denna teknik kunna få en ”ögonblicksbild” av cytokinproduktionen vid sepsis.

Resultaten av detta projekt har redovisats i delarbete III och IV.

I det tredje arbetet undersöktes sammanlagt 32 patienter med olika grader av sepsis inlagda på Karolinska universitetssjukhuset. Som kontrollpersoner användes friska frivilliga från sjukhuspersonalen (30 st.). ELISpot-metoden användes för att analysera både spontan frisättning av cytokiner från vita blodkroppar och frisättning efter retning av dessa blodkroppar med endotoxin, även kallat LPS, ett bakterieämne som vanligen orsakar en kraftig reaktion hos både immunceller och organismer. Parallellt undersöktes halten av cytokiner i blodet.

Trots att halten cytokiner i blodet var påtagligt förhöjd hos sepsispatienterna jämfört med kontrollpersonerna, var andelen cytokinfrisättande vita blodkroppar låg och likartad mellan patienter och kontrollpersoner. Vi konstaterade därför att vita blodkroppar i blodcirkulationen inte förefaller vara ursprunget till de förhöjda cytokinnivåer som uppmäts i blodet under sepsis. Dessa cytokinproducerande celler finns istället sannolikt i vävnaden och närmare beskrivning av sepsissjukdomen utifrån mätning av cytokinutsöndring från vita blodkroppar i blodcirkulationen verkar inte låta sig göras.

LPS-stimulering av de vita blodkropparna åtföljdes däremot av en betydande ökning av antalet cytokinproducerande blodkroppar hos bägge grupperna. Medan denna ökning var likartad mellan patienterna och kontrollpersonerna för två av cytokinerna (IL-6 och TNF), var den betydligt lägre för de övriga fyra undersökta cytokinerna (GM-CSF, IL-1 β , IL-12p40 och IL-10) inom sepsisgruppen. Graden av nedsatt utsöndringsförmåga vid LPS-aktivering befanns sammanhänga med sjukdomsgraden. Detta skulle eventuellt kunna användas som en markör för graden av immunhämning vid sepsis.

Sammanfattningsvis förefaller nedregleringen av cytokinfrisättning att vara selektiv vid sepsis, eftersom vissa cytokiner berörs men inte andra, samtidigt som den står i proportion till sjukdomsgraden för de nedreglerade cytokinerna.

Det fjärde delarbetet var en förberedande laboratoriestudie inför delarbete III. Här undersöktes förmågan hos vita blodkroppar att utsöndra cytokiner efter endotoxinretning. De vita blodkropparna uppdelades i de olika undergrupperna segmentkärniga (granulocyter) och enkelkärniga (monocyter och lymfocyter) celler. Det är väl känt att de enkelkärniga vita blodkropparna står för den huvudsakliga cytokinproduktionen, medan granulocyternas möjligheter till cytokinsignalering är mer omdebatterad, med motstridiga resultat från olika studier. Genom att använda ELISpot-metoden fann vi att granulocyterna hade en begränsad cytokinarsenal, bland de undersökta cytokinerna fann vi endast sekretion av IL-8, MIP-1 β och TNF. Vi konstaterade också att tidigare rapporterade granulocyt-associerade cytokiner sannolikt kan tillskrivas förorening av ett fåtal monocyter.

De enkelkärniga cellerna utsöndrade som förväntat betydligt fler cytokiner och också av en större mängd. När vi separerade monocyter från de övriga enkelkärniga cellerna fann vi att antalet cytokinutsöndrande celler reducerades med över 90 procent. Vi kunde således konstatera att monocyterna står för den absoluta huvuddelen av den cytokinutsöndrande förmågan hos vita blodkroppar.

Sammantaget visar dessa studier:

- vilka tillgängliga laboratorieprover vi bör kunna använda oss av för att på ett så tillförlitligt sätt som möjligt kunna diagnosticera allvarliga antibiotikakrävande infektioner och besläktade tillstånd hos patienter på infektionsakuten
- ett stort behov av nya diagnostiska tester för att kunna nå bättre resultat och kunna säkra en fortsatt tillgång till en hotad antibiotikaresurs
- att svår sepsis är ett vanligt tillstånd hos patienter som läggs in på infektionskliniken, men att diagnosen inte förefaller vara lika allvarlig som i andra rapporterade sammanhang
- att den s k SIRS-diagnosen bör överges
- att vita blodkroppar i blodcirkulationen inte utsöndrar de cytokiner som finns i blodet vid sepsis
- att de vita blodkropparnas förmåga att utsöndra vissa cytokiner försämras vid sepsis i proportion till sjukdomsgraden
- en närmare beskrivning av enskilda vita blodkroppars förmåga att utsöndra cytokiner

Vår förhoppning är att dessa observationer i viss mån skall kunna bidra till ett förbättrat beslutsunderlag för läkare som bedömer och behandlar allvarligt infektionssjuka patienter i sin vardag, men också utgöra grund för fortsatt forskning inom sepsisområdet.

8 ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all who contributed to the completion of this endeavour. Especially I wish to thank:

Bengt Gårdlund – associate professor, senior colleague, consultant, scientist, sepsis expert, farmer, hunter (how do you manage it all?) and friend, for your willingness to embark on this journey together with me. Along with your truly impressive knowledge of sepsis in theory and in practice, your kind support and patience have been essential for this thesis. Also, I would like to thank you for sharing enjoyable moments at congresses, in bars and in the woods. When this is over, next time in the woods, OK?

Jan Andersson – professor and outstanding scientist, for your very generous acceptance of me as your PhD student. Our meetings have always been rewarding and your brilliant brain-storming and benevolent enthusiasm have fuelled me more than I think you are aware of.

The rest of our **SAMBIO 2006** funded research group:

Christian Smedman, black belt in the ELISpot technique, for teaching me the ELISpot assay (no belt for me), for good companionship and cooperation, and for somehow making me feel like I am your age – not a bad experience. **Staffan Paulie** for discreet wisdom and helpful distinctness, and for proposing the joint project. **Lindvi Gudmundsdotter** for your kind assistance and your capacity to get things done. You catalyzed us. **Anna Somell** for good cooperation and for recruitment of patients. Last, but certainly not least, **Kopek Nihlmark**, without whom this project would never have come about. Undoubtedly one of the more profitable cocktail chats I have ever had, Kopek. Thank you for that and the following coordination of our project, which also included some relieving private conversations.

My old friend **Gunnar Sandersjö** who invited Kopek and me to the same party and provided the cocktails.

All **patients** and **volunteers** without whom this work would not have been possible.

Colleagues and **staff** at the Department of Infectious Diseases who generously donated their blood and served as healthy controls.

The **staff** at the Department of Infectious Diseases, the Emergency Department, and the Intensive Care units at Karolinska University hospital for kind assistance in enrolment procedures and sampling of blood and data. Your positive attitude and constant readiness to do a little bit more is fundamental to the clinical research, let alone the patients.

My colleague **Karin Hansson** for planning and starting the Emergency Department study together with Bengt and me.

Research nurse **Gunilla Herman** for efficient assistance with data base inclusion and patient enrolment.

Professor **Kristina Broliden** for letting us use your lab resources, for good advice and because you saw solutions where I saw problems.

The **staff** at the Haematology section of the Department of Clinical Chemistry for generously providing expertise and resources.

My mentor and friend **Li Tsai**, for being there the one time I needed you.

Mitchell Shiller, M.D. for altruistically checking the language of the greater part of this thesis manuscript. Any remaining errors are my own.

Former heads of the Department of Infectious Diseases, **Elda Sparrelid** and **Jan Carlson** for allowing me time off from clinical duties for research.

Lennart Östlund for finding rostering solutions that work in spite of the constant shortage of physicians. Pure wizardry.

Spotify for helping me endure.

Vinnova, the Swedish foundation for Strategic Research (SSF), **The Research Council (VR)**, **Bert von Kantzows foundation**, **Pfizer**, and **the regional agreement on medical training and clinical research (ALF)** for financial support.

All **senior colleagues** who taught me the tricks of the trade and served as role models – thank you.

All **colleagues** who share the ups and downs of our clinic with passion and good humour – it never gets boring.

All my many **dear friends** for distractions, inspiration and moral support, just a few of you mentioned here:

The **Gourmet** brothers, you have known me since ages and formed me as a person. By the way – who's next?

The **Tuesday floorball gang** – it's so nice to blow off some steam together with you guys.

The hunters of **Löfvik** – the serenity, the meditation, the adrenalin, the companionship. I am aching to get back.

The **Wine boys** and the **Book girls** – what a great bunch you are, our treks together are legendary. A small group of people hiking in pristine nature, philosophizing together – a primordial pleasure.

Family and relatives, for being there, for being who you are, and for letting me be who I am.

My dear mother **Kerstin** for once planting the idea that I could become a physician, and for all the marvellous support to my family over the years.

My mother-in-law **Ami** for equally superb support.

Simon, **Sebastian** and **Pelle** for making me a very proud father.

Lastly, the sun of my solar system, my mainstay, my love, my friend, my wife **Ann** for your inexhaustible patience and never-ending love.

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