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**CLINICAL AND
PATHOPHYSIOLOGICAL ASPECTS OF
SEPSIS**

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Institutet**

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Cover picture: Immunofluorescent staining of neutrophils evaluated by confocal microscopy. Dual staining of heparin-binding protein (green) and resistin (red) (cell nuclei shown in blue) reveal their co-localization (yellow).

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To my family

*"The most exciting phrase to hear in science,
the one that heralds new discoveries,
is not Eureka! (I found it!) but rather,
"hmm.... that's funny...."*

Isaac Asimov

ABSTRACT

Severe sepsis and septic shock represent challenging problems for the health care system. Despite adequate antibiotics and modern intensive care, severe sepsis is associated with a substantial mortality rate of around 30%, which rises even higher if exacerbated by septic shock, and the incidence continues to increase. In severe sepsis and septic shock, the normally tightly controlled balance between the inflammatory, coagulatory and neuroendocrine systems is lost. Our understanding of the causes, mitigating factors and mediators of severe sepsis has advanced in the last number of years. However, immunomodulatory interventions specifically directed against cytokines that all appeared promising in animal studies, did not translate well into human clinical trials. It has been suggested that the failure of many sepsis trials may in part be due to enrollment of diverse patients with sepsis of varying severity and different causative microorganisms. We, and others, believe that successful clinical trials of immunotherapeutic agents in sepsis require well defined patient cohorts with respect to severity and microbiological aetiology. This thesis project aimed to document clinical presentation and outcome of severe sepsis and septic shock, to evaluate clinical efficacy of adjunctive polyspecific intravenous immunoglobulin therapy (IVIG) in streptococcal toxic shock syndrome (STSS) and to define pathogenic mechanisms in sepsis, with a specific emphasis on the role of heparin-binding protein (HBP) and resistin; recently identified markers of severity in sepsis.

In **paper I** we conducted a prospective observational study of 101 patients with severe sepsis and septic shock. We reported a relatively low mortality in severe sepsis/septic shock, in aspects of both short- and long-term mortality, compared to studies outside Scandinavia. A troubling finding was that women received delayed antibiotics as compared to men.

In **paper II**, we documented clinical efficacy of IVIG therapy in a comparative observational study of 67 patients with STSS. This study demonstrated a significantly reduced mortality rate among STSS patients receiving IVIG as compared to patients who did not. Also clindamycin therapy was identified as an important factor for survival. The IVIG-group had a higher degree of NF as compared to the non-IVIG group.

In **paper III** and **IV**, the role of novel biomarkers in sepsis, i.e. HBP and resistin was explored *in vitro* and *in vivo*. Paper III focuses on resistin responses in STSS and necrotizing fasciitis (NF). The results demonstrate that STSS and NF are characterized by hyperresistinemia in circulation as well as at the local site of infection. Importantly, neutrophils were identified as a novel and dominant source of resistin in bacterial septic shock. *In vitro* assays using primary neutrophils showed that resistin release was readily triggered by streptococcal cell wall components and by the streptococcal M1 protein, but not by the potent streptococcal superantigens or LPS. In paper IV we explored whether neutrophil responses, in particular the release of sepsis-associated factors HBP and resistin, vary depending on bacterial stimuli and how this relates to sepsis of different aetiology. Fixed streptococcal strains induced significantly higher release of HBP and resistin, compared to *S. aureus* or *E. coli*. *In vivo* analyses of HBP and resistin in plasma of septic patients revealed elevated levels as compared to non-infected critically ill patients. HBP and resistin correlated significantly in septic patients, with the strongest association seen in group A streptococcal cases. The study reveals pronounced differences in neutrophil responses to various bacterial stimuli, and shows that streptococcal strains are particularly potent inducers of HBP and resistin.

In summary, this thesis provides new insight concerning mortality of sepsis patients in intensive care units and further supports the adjunctive treatment with IVIG in STSS patients. It also adds to the understanding of the complex pathophysiology of sepsis and our observations on bacterial induced neutrophil activation underscore the need for personalized medicine in sepsis.

LIST OF PUBLICATIONS

- I. **Linnér A**, Sundén-Cullberg J, Johansson L, Hjelmqvist H, Norrby-Teglund A, Treutiger CJ. Short- and long-term mortality in severe sepsis/septic shock in a setting with low antibiotic resistance: a prospective observational study in a Swedish University Hospital. *Frontiers in Public Health*. 2013;1:51
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- III. Johansson L, **Linnér A**, Sundén-Cullberg J, Haggar A, Herwald H, Loré K, Treutiger CJ, Norrby-Teglund A. Neutrophil-derived hyperresistinemia in severe acute streptococcal infections. *Journal of Immunology*. 2009; 183:4047-54.
- IV. **Linnér A**, Snäll J, Ibold H, Janos M, Linder A, Herwald H, Norrby-Teglund A, Johansson L. Differential neutrophil responses to bacterial stimuli: Differential neutrophil responses to bacterial stimuli: Streptococcal strains are potent inducers of heparin-binding protein and resistin-release. *Submitted manuscript*.

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Horst S.A, **Linnér A**, Beineke A, Lehne S, Høltje C, Hecht A, Norrby-Teglund A, Medina E, Goldmann O. Prognostic Value and Therapeutic Potential of TREM-1 in *Streptococcus pyogenes*-Induced Sepsis. *Journal of Innate Immunity*. 2013;5(6):581-90.

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Johansson L, Snäll J, Sendi P, **Linnér A**, Thulin P, Linder A, Treutiger CJ, Norrby-Teglund A. HMGB1 in severe soft tissue infections caused by *Streptococcus pyogenes*. *Frontiers in Cellular and Infection Microbiology*. 2014;4:4.

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LIST OF ABBREVIATIONS

ACIA	Acquired computerized image analysis
AMP	Antimicrobial peptide
APC	Antigen presenting cell
APACHE	Acute physiology and chronic health evaluation
ARDS	Acute respiratory distress syndrome
CAP37	Cationic antimicrobial protein of 37 kD
CARS	Compensatory anti-inflammatory response system
DAMP	Danger associated molecular pattern
DNase	Deoxyribonuclease
EGDT	Early goal directed therapy
ELISA	Enzyme linked immune sorbent assay
ER	Emergency room
Fab	Fragment antigen binding
FACS	Fluorescence-activated cell sorting
Fba	Fibronectin binding protein
Fc	Fragment crystallizable
GAS	Group A <i>streptococci</i>
HBP	Heparin-binding protein
HLA	Human leukocyte antigen
HMGB-1	High mobility group box 1
ICAM	Intracellular adhesion molecule
ICU	Intensive care unit
INF	Interferon
IL	Interleukin
IVIG	Intravenous immunoglobulin G
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MIF	Macrophage migration inhibitory factor
MOF	Multiple organ failure
MPO	Myeloperoxidase
MHC	Major histocompatibility complex
NET	Neutrophil extracellular trap
NF	Necrotizing fasciitis
NF- κ B	Nuclear factor- κ B
NK	Natural killer cell
NO	Nitric oxygen
NOD	Nucleotide-binding oligomerization domain
PAM	Plasminogen-binding group A streptococcal M-like protein
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood monocyte cell
PBP	Penicillin binding protein
PBMC	Peripheral blood mononuclear cell
PMN	Polymorphonuclear neutrophil
PPR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
RCT	Randomized controlled trial

RIG	Retinoic acid-inducible gene
ROS	Reactive oxygen species
SAg	Superantigen
SAPS	Simplified acute physiology score
SSC	Surviving Sepsis Campaign
Sfb	Streptococcal fibronectin binding protein
SIRS	Systemic inflammatory response syndrome
SLO	Streptolysin O
SLS	Streptolysin S
SmeZ	Streptococcal mitogenic exotoxin Z
SOFA	Sepsis-related organ failure assessment
Spe	Streptococcal pyrogenic exotoxin
SpyCEP	<i>Streptococcus pyogenes</i> cell-envelope protease
SSA	Streptococcal superantigen
SSC	Surviving sepsis campaign
STSS	Streptococcal toxic shock syndrome
SuPAR	Soluble urokinase plasminogen activator receptor
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TREM	Triggering receptor expressed on myeloid cells
VCAM	Vascular cell adhesion molecule

1 INTRODUCTION

1.1 GENERAL ASPECTS OF SEPSIS

Severe sepsis and septic shock represent major complex medical conditions that have challenged the health care system worldwide for ages. Stepping some 2000 years back in history, Hippocrates in ancient Greece used the word sepsis (σήψις; putrefaction and decomposition of organic matter) to describe a harmful event where flesh rots [1, 2]. Throughout history, the condition has puzzled and engaged the medical field. The famous researchers Pasteur, Semmelweis and others proposed the germ theory whereby sepsis or “blood poisoning”, was defined as a systemic infection resulting from invasion by pathogenic organisms. Unfortunately, the introduction of the antibiotic era did not fully solve the problem of sepsis mortality, and it was later suggested that it was the host, not the pathogen itself, that caused much of the poor outcome of sepsis [3]. Several epidemiological studies have reported high incidence in the general population that appears to increase over time. Apart from causing suffering and substantial risk of death, the condition increases burden on healthcare systems throughout the world, giving rise to extensive direct healthcare costs of hospitalization and economic costs of post-sepsis care [4, 5]. Despite adequate antibiotics and modern intensive care, severe sepsis is associated with a mortality rate of around 30% [6], which rises up to 50% if exacerbated by septic shock [7, 8]. The mortality rates differ across the world due to several factors such as age, comorbid disease burden, regional health patterns, delivery of and access to health care, as well as different genomic influences [9]. In severe sepsis and septic shock, the normally tightly controlled balance between the inflammatory, coagulatory and neuroendocrine systems is lost. Our understanding of the causes, mitigating factors and mediators of severe sepsis has advanced in the last number of years, and efforts have been made to find new potential immunotherapies that can interact with released mediators. It has been suggested that the failure of many sepsis trials besides entering “too late” in the scenery, may in part be due to treatment effects obscured by an exceedingly heterogeneous patient population with sepsis of highly varying severity caused by all too diverse microorganisms.

The main purpose of this thesis was to focus on both clinical and pathophysiological aspects using well defined patient-cohorts as well as clinically relevant *in vitro* assays. The studies aim not only to seek deeper knowledge in pathophysiological aspects and identify novel potential targets for intervention, but also to define targeted patient cohorts for future clinical trials.

1.1.1 Definitions of severe sepsis and septic shock

As for other clinical conditions, there is a need for general and easy-to-use definitions of sepsis. International recommendations of the terms sepsis, severe sepsis and septic shock were established in a consensus meeting in 1992 [10]. These definitions have gained worldwide acceptance and serve as standard criteria for enrollment of patients in clinical sepsis trials [11]. The presence of a systemic inflammatory response syndrome (SIRS) is central in the consensus definitions. At least two of the following criterion is needed for SIRS-diagnosis: fever of $>38^{\circ}$ or $<36^{\circ}$, a heart frequency of >90 beats/min, respiratory frequency of >20 breaths per minute or $\text{PaCO}_2 <4.3$ kPa, white blood cells $>12 \times 10^9/\text{l}$; $<4 \times 10^9/\text{l}$, or $>10\%$ immature forms. If it has its origin in an infection, the term sepsis should be used. Severe sepsis was defined as sepsis associated with organ dysfunction, hypoperfusion or hypotension. The term septic shock was earmarked for cases of severe sepsis with hypotension refractory to fluid replacement.

Altogether, sepsis, severe sepsis and septic shock define three progressive disease stages of increasing severity, characterized by lower incidence but increased case fatality rates (see schematic overview in figure 1).

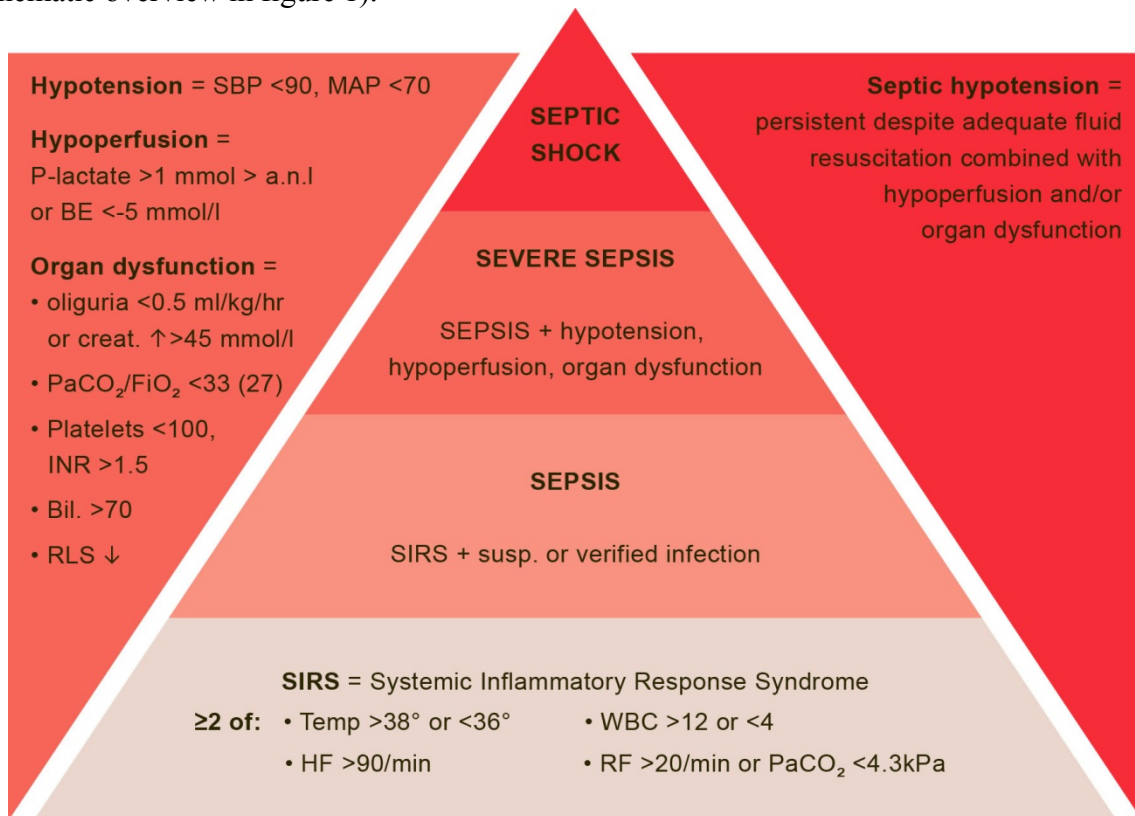


Figure 1. The simplified sepsis definitions. HF: heart frequency, WBC: white blood cells, RF: respiratory frequency, SBP: systemic blood pressure, MAP: mean arterial pressure, RLS: reaction level scale, BE: base excess, a.n.l: above normal limit, Bil: bilirubin. Modified with permission from MD M. Brink, Sahlgrenska Hospital, Sweden [12].

It is important to be aware of the fact that the SIRS criteria are sensitive; up to 90 % of patients admitted to the intensive care unit (ICU) meet the criteria which can be caused by many non-infectious clinical conditions like trauma, burn injuries etc. [13, 14]. In order to facilitate the diagnosis of sepsis, a second consensus meeting took place in 2001 [15], in which the original SIRS criteria expanded into several parameters listed in Table 1. The update in 2001 made the sepsis description more complete, at the same time as it made the diagnosis more difficult to interpret for the clinician. Suggestions have been made to change the definitions in order to facilitate for the clinician [16], and as for the enrollment of patients in sepsis trials, the original 1991 sepsis criteria is used.

Bacteremia denotes the presence of viable bacteria in the blood but since the sensitivity of current microbiological methods is poor, it is too narrow a definition for sepsis in which infection is presumed, but often not proven. Similarly, *septicemia*, the presence of microorganisms or their toxins in the blood, is no longer used in order to avoid misunderstandings.

Table 1. *Diagnostic criteria for sepsis, severe sepsis and septic shock**

<p>Sepsis: documented or suspected infection plus ≥ 1 of the following:</p> <p>General variables</p> <ul style="list-style-type: none">Fever (core temperature, $>38.3^{\circ}\text{C}$)Hypothermia (core temperature, $<36^{\circ}\text{C}$)Elevated heart rate (>90 beats per min or >2 SD above the upper limit of the normal range for age)TachypneaAltered mental statusSubstantial edema or positive fluid balance (>20 ml/kg of body weight over a 24-hr period)Hyperglycemia (plasma glucose, >120 mg/dl or 6.7 mmol/liter) in the absence of diabetes <p>Inflammatory variables</p> <ul style="list-style-type: none">Leukocytosis (white blood cell count, $>12 \times 10^9/\text{l}$)Leukopenia (white blood cell count, $<4 \times 10^9/\text{l}$)Normal white blood cell count with $>10\%$ immature formsElevated plasma C-reactive protein (>2 SD above the upper limit of the normal range)Elevated plasma procalcitonin (>2 SD above the upper limit of the normal range) <p>Hemodynamic variables</p> <ul style="list-style-type: none">Arterial hypotension (systolic pressure <90 mm Hg; mean arterial pressure <70 mm Hg; or decrease in systolic pressure of >40 mm Hg in adults or to >2 SD below lower limit of the normal range for age)Elevated mixed venous oxygen saturation $>70\%$Elevated cardiac index (>3.5 liters/min/m^2 of body-surface area) <p>Organ dysfunction variables</p> <ul style="list-style-type: none">Arterial hypoxemia ($\text{PaO}_2/\text{FIO}_2 <300$ mm Hg or <40 kPa)Acute oliguria (urine output, <0.5 ml/kg/hr or 45 ml/hr for at least 2 hr)Increase in creatinine level of >0.5 mg/dl (>44 $\mu\text{mol/liter}$)Coagulation abnormalities (international normalized ratio, >1.5; or activated partial-thromboplastin time, >60 sec)Paralytic ileus (absence of bowel sounds)Thrombocytopenia (platelet count, $<100,000/\text{mm}^3$)Hyperbilirubinemia (plasma total bilirubin, >4 mg/dl [68 $\mu\text{mol/liter}$]) <p>Tissue perfusion variables</p> <ul style="list-style-type: none">Hyperlactatemia (lactate, >1 mmol/liter)Decreased capillary refill or mottling <p>Severe sepsis: sepsis plus organ dysfunction, hypoperfusion or hypotension</p> <p>Septic shock: sepsis plus refractory¹ hypotension or hyperlactatemia</p>

**Adapted from Levy et al.[15].*

¹ *Refractory hypotension is defined as either persistent hypotension or a requirement for vasopressors after the administration of an intravenous fluid bolus.*

1.1.2 Epidemiology

Due to a difference in local settings and diversity in the use of recommended definitions, there is a great variation in sepsis incidence throughout the world, and the full magnitude and impact of sepsis is not known [17]. In an often cited study by Angus et al, the total estimated number of severe sepsis cases exceeds 750,000 per year in the United States (300 per 100,000 inhabitants) [18]. In this study, the projected increase of incidence was 1.5% per year based on the growth and aging of the U.S. population. Subsequent studies have shown this estimate

of raising incidence to be low, with increases in sepsis rates reported to be as high as 9% per year [19, 20], a number that was recently reported to be even higher [21]. This could in part be due to ageing and increasing co-morbidities amongst the patients, but it could also be due to increased awareness and surveillance of sepsis [22, 23]. The new *International Classification of Diseases, 9th Revision* (ICD-9) coding rules for sepsis, and the potential confusion over the distinction between septicemia and severe sepsis are also confounders in the reporting and interpretation of modern trends [23, 24]. In the United States, severe sepsis is recorded in 2% of patients admitted to the hospital, of which 50% are treated in the ICU. This represents 10% of all ICU admissions and a reported 215,000 deaths annually [18]. The reported incidence is higher than for many other more well-known diseases like breast cancer (110/100,000), colorectal cancer (50/100,000) and AIDS (17/100,000) [25]. The incidence of sepsis in Europe was reported to be 200/100,000, severe sepsis 100/100,000 and septic shock 50/100,000 [26]. In northern Europe the numbers reported are lower, with an incidence of sepsis in Norway of 149/100,000 inhabitants [27] and severe sepsis in Finland of 38/100,000 [28]. In Sweden the incidence is not fully known, but according to registers of the Swedish National Board of Health and Welfare, the incidence of severe sepsis is estimated to 200 per 100,000 inhabitants and septic shock is estimated at 30 cases per 100 000 inhabitants, most likely underreported. Unpublished Swedish national studies of community-acquired severe sepsis shows a yearly incidence of 210 per 100 000 inhabitants, which gives 19 000 cases per year [29]. According to the Swedish death case register, approximately 1000 patients succumb from sepsis every year. The incidence of severe sepsis outside modern societies is largely unknown. Adhikari et al. [30] estimated an incidence of up to 19 million cases worldwide per year, by extrapolating from treated incidence rates in the United States. However, the true incidence is most likely even higher.

Risk factors for severe sepsis and septic shock are many and well-known, including underlying health status (e.g., cancer, the acquired immunodeficiency syndrome and chronic obstructive pulmonary disease), age and the use of immunosuppressive therapy [18]. In characterizing genetic predisposition, many studies have focused on polymorphisms in genes encoding proteins important in the pathogenesis of sepsis, including cytokines and other mediators involved in innate immunity, coagulation, and fibrinolysis, with inconsistent findings [31, 32].

1.1.3 Aetiology

Bacteria cause the majority of sepsis cases, fungal and viral origin being less frequent [33]. Previous epidemiologic studies revealed that Gram-positive compared to Gram-negative bacteria have become the most common cause of sepsis in the past 25 years (estimates in sepsis gives 200,000 cases of Gram-positive sepsis each year vs 150,000 cases of Gram-negative sepsis) [19]. In a more recent study involving 14,000 ICU patients in 75 countries, Gram-negative bacteria dominated, followed by Gram-positive bacteria and fungal infection. [34].

Blood cultures are positive in only approximately one third of the cases [35], and the most common bacteria causing community-acquired sepsis are *E. coli*, *S. aureus* and *S. pneumoniae*. Other important pathogens are group A *Streptococcus* (discussed in detail in section 1.2), *Meningococcus*, *H. Influenza*, *Klebsiella* species and *Pseudomonas aeruginosa*. In hospital acquired nosocomial sepsis, we find the addition of other bacteria, for example *Enterobacteriaceae* and *Enterococcus* [36-38]. Often the above mentioned pathogens present with distinct clinical features or symptoms due to respiratory, urogenital, skin or abdominal

origin of infection. However, in the critically ill patient, the clinical features are often similar, regardless of aetiology. This is likely due to the fact that it is rarely the bacteria (or virus or fungi) themselves that cause the symptoms, but instead an over-exaggerated host immune response to the microbial stimuli, further explained in detail in later sections. The site of origin remains stable, with respiratory infections being the most common cause of sepsis, accounting for approximately half of all cases of sepsis followed by genitourinary and abdominal sources of infection [19, 34, 39, 40].

1.2 GROUP A STREPTOCOCCAL INFECTIONS

1.2.1 Clinical aspects

As mentioned above, severe sepsis and septic shock are conditions presenting with high mortality rates. We have focused much of our research on the β -hemolytic group A *streptococcus* (GAS), a pathogen associated with one of the highest mortality rates in immune competent individuals despite the fact that the pathogen itself is fully susceptible to commonly used antibiotics. The pathogen, also referred to as *Streptococcus pyogenes*, is a strictly human bacterium that causes a wide array of diseases, from mild skin infections and upper respiratory tract infections to invasive fulminant life threatening conditions such as streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis (NF) [41, 42]. Populations-based data shows that GAS is an important cause of morbidity and mortality as it is estimated to be the ninth most common source of global mortality due to a single pathogen [43, 44], with an incidence rate between 2 and 4 per 100,000 [45]. In developing countries, the incidence rates are estimated to be even higher (>10 per 100,000) [43]. An increased incidence of these severe infections has been reported world-wide since the mid 1980s, accounting for more than 150 000 deaths annually [43, 46].

The dramatic nature of invasive GAS infections has attracted considerable attention and concern. Despite modern intensive care and prompt adequate antibiotic therapy, they are associated with high mortality rates [47]. The overall case fatality rate is as high as 50 % if STSS is present [48]. The classification of GAS infections was defined by a working group on severe streptococcal infections in 1993 [49]. The major groups are STSS, other invasive infections (including meningitis, peritonitis, pneumonia, puerperal sepsis, osteomyelitis, septic arthritis, NF, surgical wound infections, cellulitis), scarlet fever and non-invasive infections (mucous membrane and cutaneous). There are also non-suppurative complications including acute rheumatic fever and acute glomerulonephritis. These post-streptococcal conditions are more commonly found in developing countries, where rheumatic heart disease is considered the most common cardiac disease in children and young adults [43].

1.2.1.1 *Streptococcal toxic shock syndrome and necrotizing fasciitis*

STSS was first recognized in the mid 1980s after reports of increased mortality due to GAS bacteremia, and a syndrome of toxic shock, multi-organ failure and rapidly progressive soft tissue infection was observed. The classification of STSS infections was defined by a working group in 1993 (table 2) [49].

NF, a rapidly advancing infection of the subcutaneous tissues and fascia with necrosis and relative sparing of the underlying muscle, was early thought to be a necessary part of the diagnosis, but is now considered as a separate entity (figure 2). A hallmark symptom of NF is pain, often in combination with localized swelling and erythema. Up to 50% of the GAS-associated NF cases develop STSS [50], and in these cases, the mortality rates increase and can often be as high as 60% [50, 51]. STSS commonly occurs in association with an infection of a cutaneous lesion, but in 50% of the cases the port of entry is unknown [46]. In these cases, a transient bacteremia from oropharynx has been suggested as a source of origin. A risk factor for developing invasive GAS infection is a lack of protective antibodies against superantigens, discussed in a later section [52, 53].

Table 2. *Classification of STSS**

I.	Isolation of group A streptococci from a:
	A. Normally sterile site – definite case
	B. Non-sterile – probable case
II.	Hypotension
III.	≥2 of the following signs:
	A. Renal impairment
	B. Liver involvement
	C. Generalized erythematous macular rash that may desquamate
	D. Coagulopathy
	E. Soft tissue necrosis
	F. Adult respiratory distress syndrome

* *Adapted from Working group of GAS, JAMA, 1993 [49].*

The cornerstones of management of STSS and NF are antimicrobial therapy and surgery. GAS is fortunately uniformly susceptible to both benzyl-penicillin and other β -lactam antibiotics, however in severe invasive infections like STSS and NF, the addition of clindamycin is associated with improved outcome [54, 55]. The most important reason for using the combination is that it addresses the inoculum effect. In summary, targets for β -lactam antibiotics are the penicillin binding proteins (PBP), expressed during the log-phase of the growth of the bacteria. With a large inocula (the case in severe infections), the bacteria may reach a stationary phase of growth, where the PBPs are down-regulated. This phenomena is referred to as the Eagle effect, originally describing the paradoxically reduced antibacterial effect of penicillin at high doses [56]. Today it describes the relative lack of efficiency of β -lactam antibiotics on infections with a large number of bacteria [57]. More specifically, clindamycin also inhibits protein synthesis, including M protein and superantigens [58-61]. In light of these biological mechanisms, the additional use of clindamycin is believed to improve outcome. Moreover, early surgical debridement is recommended if NF is present, with immediate debridement of affected and surrounding tissues [62]. Another approach is to use hyperbaric chambers which facilitate blood supply to necrotic tissue through the use of hyperbaric oxygen [63]. The administration of adjunctive polyspecific immunoglobulin (IVIG) therapy has been suggested to stabilize the patient, allowing a more conservative surgical approach, thereby reducing the risk of extensive tissue debridement in the severe ill patient [64]. The IVIG therapy is discussed in detail in section 1.4.5.

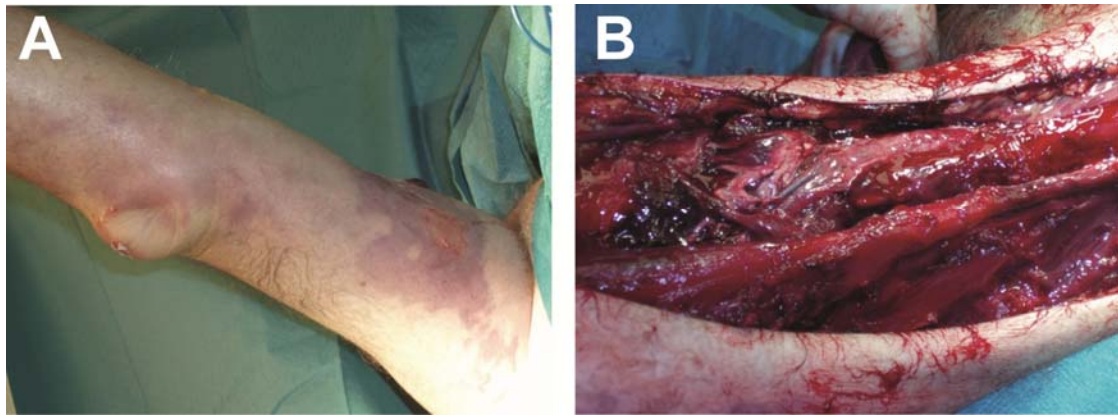


Figure 2. *Necrotizing fasciitis before and after surgical procedures. A. A 55-year old male with a minor lesion of the right elbow three days prior, presents at the emergency room with bursitis and severe pain. A preliminary diagnosis of STSS in combination with NF was observed, and the patient received antibiotic treatment with β -lactams and clindamycin, in combination with IVIG therapy. B. Visualization of the surgical procedures that followed where 75% of the triceps had to be removed. Blood cultures revealed growth of GAS (Photo with the permission from MD P. Thulin, Sahlgrenska Hospital, Sweden).*

1.2.2 Pathogenetic aspects

GAS has more than 60 properties of complex virulence factors which make it very potent as a human pathogen [65]. The virulence factors are either cell-associated, or secreted, and interact with the host in different ways; to promote survival of the bacteria by resisting phagocytosis; continuous growth, adherence to epithelial cells and internalization into cells; and dissemination and systemic toxicity [66]. The interaction between GAS virulence factors and the host immune cells gives rise to various inflammatory responses including the severe cases of tissue injury and septic shock [41, 67]. The pathogenesis of invasive GAS is complex and many players are involved, summarized below in figure 3.

Many membrane-bound molecules of GAS are important for bacterial adherence to the host cells and tissues as well as evasion of phagocytosis. Most of the GAS isolates from severe infections have a capsule composed of hyaluronic acid, with an antiphagocytic effect [68]. Since the capsule is identical to the hyaluronic acid of the connective tissue of the host it is not immunogenic, allowing the bacteria to disguise themselves with an immunological 'self' profile. The cell wall consists of peptidoglycan with lipoteichoic acid (LTA). LTA binds to the host cells secreted fibronectin [69, 70] and facilitates adherence to epithelial cells. The C5a peptidase is a protein present on the surface of all strains of GAS. Besides an adhesive role, this protein cleaves and inactivates human C5a, which is an important chemo-attractant of phagocytic cells [66]. Another outer membrane-bound virulence factor of GAS is the streptococcal fibronectin binding protein 1 (Sfb1). Sfb1 have specific domains for binding to discrete regions of the human fibronectin molecule [71], and it has been shown to mediate internalization into nonphagocytic cells [72] and adherence to skin, throat and respiratory epithelial cells [73]. Fba-A is another surface-associated fibronectin binding protein present in GAS that can bind to host fibronectin and play an important role in the bacterial invasion of epithelial cells [74].

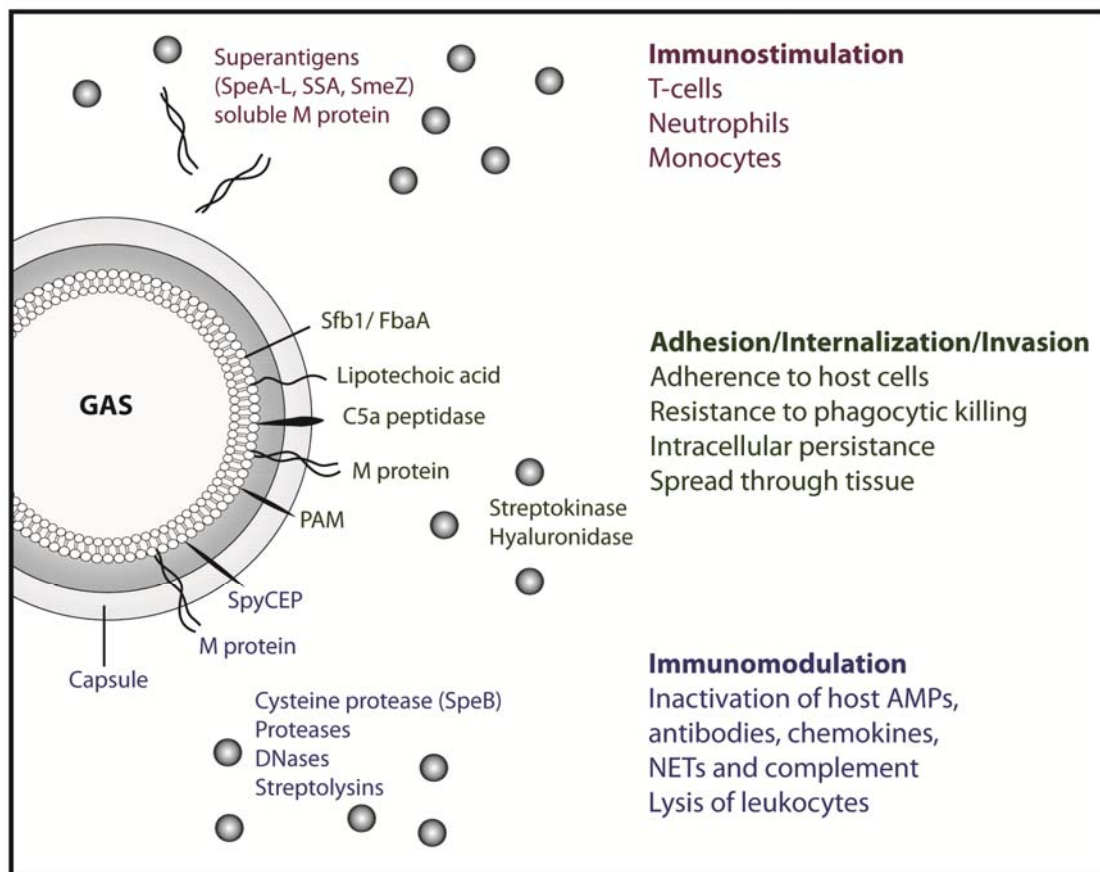


Figure 3. Virulence factors of GAS. Immunostimulatory factors, factors important for bacterial adhesion and invasion, and immunomodulatory factors. AMPs, antimicrobial peptides; NETs, neutrophil extracellular traps; PAM, plasminogen-binding GAS M-like protein; SmeZ, streptococcal mitogenic exotoxin Z; Spe, streptococcal pyrogenic exotoxin; SSA, streptococcal superantigen; SpyCEP, *Streptococcus pyogenes* cell-envelope protease. The figure is modified from Johansson et al. [75].

Yet another important virulence factor is SpyCEP, *streptococcus pyogenes* cell-envelope protease, a cell wall anchored IL-8 protease. SpyCEP cleaves and inactivates the neutrophil chemoattractant IL-8 and other chemokines, thereby disrupting neutrophil recruitment to the site of infection, as well as neutrophil-mediated GAS killing [76, 77]. SpyCEP activity is correlated with the severity of invasive disease among GAS isolates, regardless of *emm* type [77, 78]. It has also been shown to be essential for systemic spread of invasive GAS after intramuscular infection [79].

The majority of the secreted proteins contribute to spread and growth of the bacteria, as well as causing tissue destruction and systemic toxicity [80]. Also, they are able to inactivate host complement factors and antimicrobial peptides, antibodies, chemokines and neutrophil extracellular trap (NET) formations. For example, haemolysins are molecules that can lyse erythrocytes, PMNs and platelets by forming pores in their cell membrane. GAS produces two haemolysins; streptolysins O (oxygen labile) and S (serum soluble). Streptolysin O (SLO) can be found in many pathogenic bacteria with toxic effects and the ability to induce apoptosis of macrophages [81]. SLO can also induce platelet and neutrophil aggregation, suggested to contribute to the vascular dysfunction in severe GAS infections [82]. Streptolysin S (SLS) is responsible for the β -haemolysis around colonies on blood agar plates, and both SLO and SLS

are able to damage the membranes of neutrophils [66]. Hyaluronidase is released in order to degrade hyaluronic acid, the basic substance of host connective tissue, facilitating the spread of infection along fascial planes. Streptokinase, also known as fibrinolysin, is another spreading factor of GAS, which can activate host's plasminogen. Once host plasminogen is bound to the surface-expressed plasminogen-binding site on the bacteria, it is activated to plasmin by bacterial expressed streptokinase [66]. Plasmin degrades the fibrin blood clot and hinder the build-up of fibrin barriers, and as a result, soft tissue infections due to GAS are more diffuse, and often rapidly spreading, than the well localized abscesses that typify staphylococcal infections [83]. In addition, the individual difference in binding host derived plasminogen to the bacteria and activation to plasmin has been suggested to promote a localized infection into a more severe disease [83]. GAS can also produce deoxyribonucleases (DNases), enzymes that hydrolyze nucleic acids, facilitating the spread through tissues [66]. DNase also allows the bacteria to escape from the DNA net released from phagocytes [84]. SpeB, which is a potent cysteine proteinase, is capable of cleaving and inactivating many host proteins, such as the cathelicidin antimicrobial peptide LL-37, fibronectin and immunoglobulins, thereby inactivating the innate effector molecules and promoting bacteria spread [66, 85].

For the purpose of my thesis, the immunostimulatory effects of the M1-protein and the superantigens are described more in detail below, since they are both known to elicit the classical clinical picture seen in STSS.

1.2.1.1 M protein

One of the major virulence factors of GAS is the M protein which is expressed on the surface of the bacteria and encoded by the *emm* gene (figure 4). The hyper-variability of the outer part of the M protein has been used for a serological typing method of GAS [86]. More commonly used worldwide nowadays is the sequencing of the hyper-variable part of the *emm* gene encoding the M-protein [87], and to date more than 150 different *emm* types have been identified [88]. Epidemiological studies of invasive GAS infections in the late 1980s shows that the majority of the outbreaks reported in different countries mainly and predominantly where caused by the GAS strains of serotypes M1 and M3 [89]. Other national surveillance studies also show that M1 and/or M3 are common in the community, however, a later study revealed M89 dominating in invasive GAS infections [90]. The prevalence of different M-types in community have been shown to vary, both over time and with the geographic area studied. There is a genetic polymorphism in the gene encoding the M protein and the most distal part of the protein shows extensive variability among strains. As a consequence, individuals may suffer from reinfection with GAS strains expressing different M-types due to a lack of specific M-type antibodies. This lack of protective immunity to specific virulence factors likely affects host susceptibility to infection. In the study by Basma et al. invasive cases had significantly lower levels of host specific protective antibodies compared to age-matched geographic controls, demonstrating that lack of protective antibodies is a risk factor to develop invasive GAS infection [52].

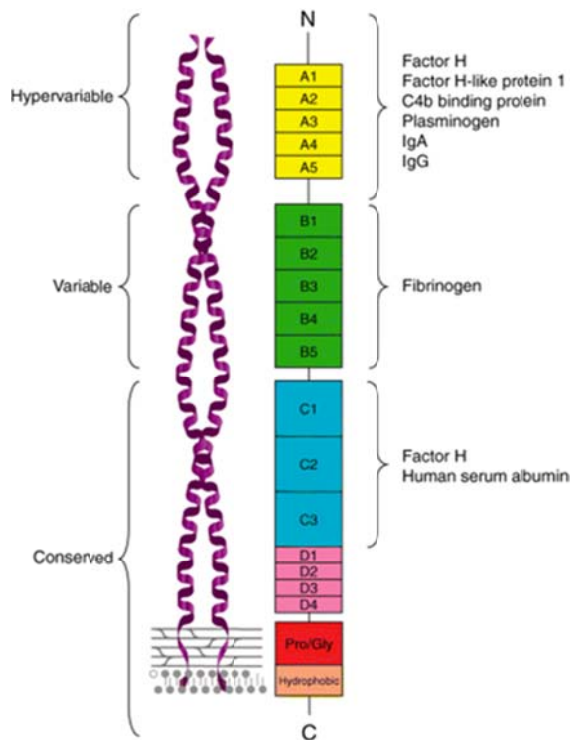


Figure 4. The dimeric alpha-helical coiled-coil structure of the M protein of the cell surface of GAS. M-typing or emm-typing is provided by the N-terminal hyper-variable part that differs between different M-types. The C-terminal part is associated with the cell wall and the membrane, and highly conserved [66, 91]. Adapted from McArthur et al. [88], with permission from Wiley and sons.

The M and M-like proteins are multifunctional factors that interfere with the host in many different ways. As GAS invades the blood during an infection, the M/M-like proteins bind to various proteins of the host, including plasminogen, albumin, immunoglobulin G and A, the proteinase inhibitor α_2 -macroglobulin and factor H and C4b-binding protein (regulatory factors from the complement system). The ability of the M proteins to resist phagocytosis is partly due to the negatively charged N-terminus of the outer part of the M-protein, giving rise to an electrostatic repulsion of the phagocytic white blood cells [92]. Also, binding to host factor H, a regulatory protein, the M-protein can protect its conserved regions from activated complement system by destabilizing the important opsonin C3b when deposited on the bacterial surface [93, 94]. The C4b-binding protein inhibits surface complement deposition by stimulating degradation of both C4b and C3b [95, 96]. On the other hand, intracellular survival of GAS in macrophages has been shown to contribute to persistence during deep tissue infections [97]. Studies also revealed that M1-protein impairs lysosomal fusion in both neutrophils and macrophages; thereby, supporting intracellular survival in these phagocytic cells [98-101].

When surface-attached, the M protein confers anti-phagocytic activity as described above. In addition, the M protein also exists in a shed form due to cleavage from the streptococcal surface by neutrophil proteases or bacterial-derived cysteine proteases such as SpeB (figure 5). Soluble M protein has been reported to have many pro-inflammatory activities, both as a toll-like receptor (TLR)-2 ligand, in forms of a superantigen, with the subsequent monocyte expression of the cytokines IL-6, IL-1 β , and tumor necrosis factor (TNF)- α [102], as well as a potent inducer of T cell proliferation with the release of Th1 type cytokines as tumor necrosis factor (TNF)- β and interferon (INF)- γ [103]. The soluble form of M protein can also bind to fibrinogen [104, 105]. The binding to fibrinogen allows the formation of complexes that subsequently bind to adhesion molecules on the neutrophil surface (β_2 -integrins). Herwald et al. showed that the M1 protein-fibrinogen complex ligate neutrophilic β_2 -integrins resulting in cellular activation and discharge of HBP from secretory vesicles [106], a potent inducer of vascular leakage, discussed in later section (figure 5). Once activated, the neutrophils adhere to the endothelium of the cell vessel walls and degranulate, releasing a wide variety of hydrolytic

enzymes. The resulting damage to the underlying endothelium leads to vascular leakage and hypercoagulability, which in turn cause the hypotension, disseminated intravascular coagulation, and organ damage that are characteristics of STSS [107].

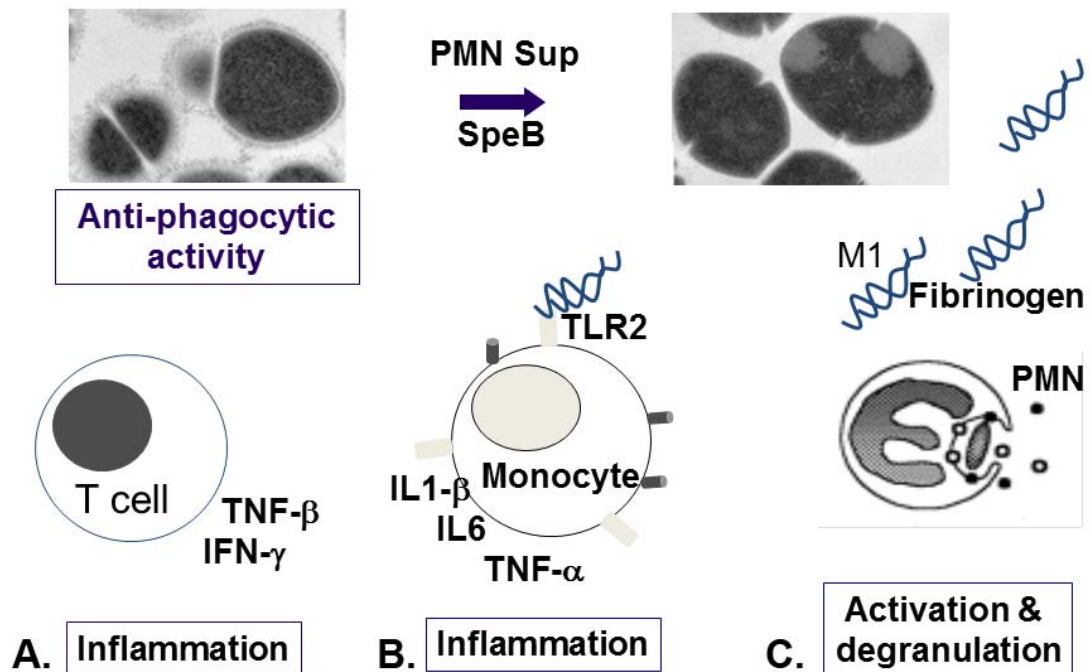


Figure 5. The M1-protein is a multipotent and powerful inducer of inflammation. When surface-attached, the M protein confers anti-phagocytic activity. It can also exist in a shedded form due to cleavage from the streptococcal surface by neutrophil proteases or bacterial derived cysteine proteases (SpeB). In A, the M1 protein is a potent inducer of T cell proliferation with the release of Th1 type cytokines as TNF-β and INF-γ. In B, the M protein interacts with TLR2 on monocytes. As a consequence, monocytes express the cytokines IL-6, IL-1β, and TNF-α. In C, M1 protein-fibrinogen complex ligate neutrophilic β₂-integrins resulting in cellular activation and discharge of HBP from secretory vesicles. Based on the articles of Pählman et al. and Herwald et al. [102, 103, 106].

1.2.1.2 Superantigens

The superantigens (SAGs) are a family of proteins able to induce a very potent inflammatory response [108]. The SAGs include the streptococcal pyrogenic exotoxins (Spes) A, C, G-M, the streptococcal superantigen (SSA) and the streptococcal mitogenic exotoxin (SmeZ) [109, 110]. Under normal conditions, antigens are processed into small peptides by the antigen-presenting cells (APC) and displayed on the surface of these cells bound as complexes to major histocompatibility complex (MHC) class II molecules. The MHC-peptide complex is thereafter recognised by T cells via T cell receptors (TCR), inducing T cell activation and further release of inflammatory cytokines. In contrast, the SAGs are able to induce T cell activation without prior processing by a direct interaction with MHC class II molecules on APCs and the Vβ domains of T-lymphocyte receptors on T cells [108]. This cross-linking results in a massive T-cell activation, and the release of excessive levels of pro-inflammatory cytokines, such as IL-1, IL-2, TNF-α, TNF-β and INF-γ [108, 111]. SAG-induced cytokines

are important contributors to the hypotensive shock and organ failure seen in patients with severe streptococcal disease like STSS and NF [112-114]. STSS is characterized by a tremendous activation of the immune system in response to the many virulence factors of GAS, resulting in the production of high levels of inflammatory cytokines [67] and giving rise to a massive vascular leakage leading to hypovolemic hypotension and multi-organ failure. Inter-individual immunogenetic differences in forms of human leukocyte antigen (HLA) class II allelic variation is seen to correlate with the severity of invasive GAS infection [114]. The risk HLA class II haplotypes have been shown to promote significantly stronger SAg-induced T cells responses as compared to the protective HLA class II types, which is in agreement with the stronger cytokine responses evident in the more severe cases [114]. In conclusion, superantigens and M protein represent major virulence factors contributing to pathogenesis of severe invasive GAS infections such as STSS and NF. Individuals that lack protective antibodies are more susceptible of invasive infection [52]. However, the severity of disease is determined by host genetic factors [114].

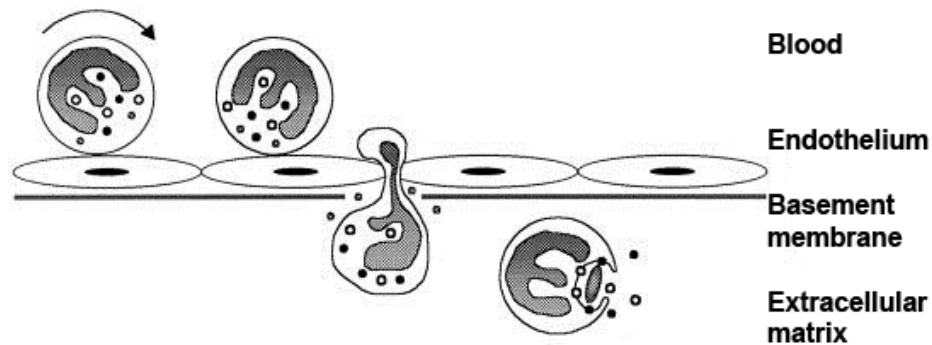
1.3 THE IMMUNE SYSTEM

To fully comprehend the pathogenesis in severely ill patients, the pathways of the “normal” immune system and its defense mechanisms vs infections need to be understood. Traditionally, the immune system is divided into two parts, the innate and the adaptive system [115]. The innate system depends on pattern recognition and constitutes the first line of defense against pathogens. It is phylogenetically well conserved and present in all multicellular organisms [116]. This immune system (including the complement system, the coagulatory system, the fibrinolytic system, cytokines, antimicrobial peptides and acute phase proteins), can be readily mobilized within minutes or hours of infection and serves to attack and eliminate the microbes invading the body. It has, however, no memory function, and thus always reacts the same way to a given stimulus. Monocytes, macrophages, neutrophilic granulocytes, natural killer cells (NK) and mast cells are all actors of the innate immune system. The adaptive, humoral (i.e. soluble in body fluids) immune system on the other hand, is present only in vertebrate organisms and serves as a second line of defense, with a memory function, responding more powerfully after each new exposure to a particular pathogen. The cells of this system are the B- and T- lymphocytes and APCs [115]. The innate system also has the ability to activate the slower adaptive immune system. In the studies in this thesis we have focused largely on innate immune responses, in particular neutrophil responses. What, then, are neutrophils?

1.3.1 Neutrophils

White blood cells or leukocytes can be divided into mononuclear cells and polymorphonuclear cells (PMN), or granulocytes. Under normal conditions the neutrophils account for 40 to 70 % of the leukocytes in human peripheral blood. In sepsis, neutrophils act as the frontline soldiers against pathogens, harboring impressive weaponry to kill invading pathogens. Phagocytosis and the release of soluble anti-microbials from granules are important mechanisms. Neutrophils are also capable of entrapping bacteria in ejected DNA-based structures containing anti-bacterial proteins such as elastase, cathepsin G, and myeloperoxidase, which have been named neutrophil extracellular traps (NETs) [117-119]. It has been shown that the NET formation can precede phagocytosis, and NETs seem to be able to encounter and trap far more bacteria simultaneously than phagocytosis alone [120, 121].

Several studies have shown that neutrophil responses in sepsis are aberrant with respect to survival, migratory capacity and functionality [122, 123]. A distinct feature of infection and sepsis is the recruitment of immature neutrophils from the bone marrow into the circulation. In addition, activated tissue macrophages and endothelial cells express TNF, IL-1, IL-8 and nitric oxide (NO), which attract neutrophils. A complex process is initiated involving adhesion of circulation neutrophils to the activated endothelium of the vessels, followed by the extravasation and migration of neutrophils to the place of tissue injury and then the elimination of microbes through phagocytosis, generation and rapidly release of reactive oxygen species (ROS), and degranulation and release of microbicidal molecules (figure 6).



Secretory vesicles	Primary granules (azurophilic)	Secondary granules	Tertiary granules
HBP CR1 CD 14 CD 11b/CD 18 Pr 3	HBP Cathepsin G Elastase CD11b/CD 18 BPI Defensins MPO Resistin	Lactoferrin Cathelicidin Collagenase CD11b/CD18 Lysozyme hCAP-18 LL-37 NGAL	Gelatinase CR1 Lysozyme



Order of release of vesicles and granules

Figure 6. *The order of release of proteins in vesicles and granules in neutrophil-mediated inflammatory response. Upon stimulation with bacterial virulence factors, the neutrophils are activated and release specific vesicles and granules to the phagocytic vacuole or the exterior of the cell. The stimulated vascular endothelium adjacent to sites of inflammation secretes neutrophil-activating substances and selectins, allowing the binding of the circulating neutrophils to the endothelium via specific selectin ligands. A firm adhesion to the endothelium is established, enabling the neutrophils to roll along the endothelium in order to eventually transmigrate the vascular basement membrane to the site of infection. Modified from Faurschou et al. 2003 [124].*

Neutrophils store a reservoir of different proteins and proteases, as well as membrane-bound receptors for endothelial adhesion molecules, extracellular matrix proteins and soluble mediators of inflammation. Most of the above described steps followed by neutrophil-activation

are dependent and due to the mobilization of cytoplasmic granules and secretory vesicles stored within the neutrophils. They enable the destruction of internalized pathogens, but also they cause local tissue damage [123]. The neutrophils contain more than 300 different proteins released in a hierarchical order during the movement of the neutrophils from the blood stream to the tissue of infection [124-127], as visualized in figure 6. The neutrophil granules can be classified as primary, secondary and tertiary. The primary granules are formed in the promyelocytic stage, and the secondary granules form in the myelocytic stage. In a later stage, called the metamyelocytic stage in which the neutrophils differentiate further, the tertiary granules are formed. The stages of development determine the specific granule contents in different granula. In addition to the granules, the secretory vesicles are characterized by their immediate release when contact is established between the neutrophil and the endothelium [128]. Upon activation of the neutrophils, the secretory vesicles translocate and cover the neutrophil surface membrane with β -integrins (membrane-associated receptors) which mediate endothelial adhesion and initiate transmigration of the neutrophils through the endothelium [129]. The controlled mobilization of the neutrophils and the regulated exocytosis of granules and vesicles permit transformation of the neutrophil from a passively circulating cell to a key effector cell of our innate immunity, enabling the neutrophil to deliver its proteins in a targeted manner.

A recent report found circulating neutrophils in sepsis patients to have a suppressed apoptosis, a longer life-span and pro-inflammatory phenotype with increased TNF- α /IL-10 ratio [130]. In most cases of severe sepsis and septic shock, the total number of circulating neutrophils increases, though some patients in contrast have low number or immature forms of neutrophils. It seems likely that rather than the total number of neutrophils, it is a subset of neutrophils that plays an important role and is engaged in tissue insult, since even in neutropenic patients, immature neutrophils degranulate and cause neutrophil-mediated lung injury [123].

1.3.2 The inflammatory response in sepsis

What happens then when microbes invade the barriers of the body? The immune system was developed in order to target microbes that enter the human body through different pathways (the skin, the mucosal membranes etc). Very rapidly, the first line of defense of the innate immune system is activated. This event is initiated through pathogen-associated molecular patterns (PAMPs), evolutionary conserved outer components or patterns on the surface of the microbes, such as the endotoxin lipopolysaccharide (LPS) of the Gram-negative bacteria, and peptidoglycan and lipoteichoic acid of the Gram-positive bacteria etc. The PAMPs are discovered by immune cells (such as neutrophils and macrophages) by pattern recognition receptors (PRR) on their surface. TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors and retinoic acid-inducible gene-1 (RIG-1)-like receptors are examples of PRRs. When PRRs recognize PAMPs, an intracellular signal-transduction pathway activates and releases transcription factors like nuclear factors- κ B (NF- κ B) – thereby inducing transcription of genes coding for numerous cytokines like TNF, IL-1, IL-6 and an initiation of the innate immune response. In addition, the PRRs sense endogenous molecules released from the dying cells, the danger associated molecular patterns (DAMPs), or alarmins. The alarmins (for example high mobility group box 1 (HMGB-1) protein and S100 proteins) are also released during sterile injury such as trauma, giving rise to the concept that the pathogenesis of multiple organ failure in sepsis is not totally different from that in noninfectious critical illness [131]. In conclusion, both PAMPs and DAMPs elicit the inflammatory response seen in sepsis. Thus, a cascade of various complex processes is initiated: phagocytosis of the microbes,

release of mediators, activation of the complement system and the coagulation cascade, release of cytokines and other mediators etc. (figure 7).

The neutrophils are now fully activated and there is a recruitment of other immune cells like the NK cells, the mast cells and eventually the endothelial cells along the vessels. The activated neutrophils starts to migrate to the tissue injury site and this, in combination with capillary leakage and increased vascular permeability, gives rise to classical host inflammatory signs (rubor, calor, dolor, tremor) and physiological signs of sepsis like vasodilatation, impaired perfusion, organ dysfunction and micro thrombosis due to an overactive coagulation system (figure 7). Eventually all these events generate an impaired oxygen delivery to all organs. This cellular hypoxia leads to anaerobic metabolism which generates metabolic acidosis and lactate accumulation, and, in combination with above events, multiple organ failure (MOF) develops. The impaired oxygen-exchange in the lungs can lead to development of acute respiratory distress syndrome (ARDS), leading to even further impairment of oxygen delivery. Early in sepsis a hyper dynamic phase is noted with increased heart rate and low systemic vascular resistance. If the hypovolemia is not corrected, or the patient's heart does not cope with these tensions, the cardiac output decreases and the circulation moves on to a hypo dynamic phase with engagement of the sympathetic nervous system and eventually the development of irreversible shock.

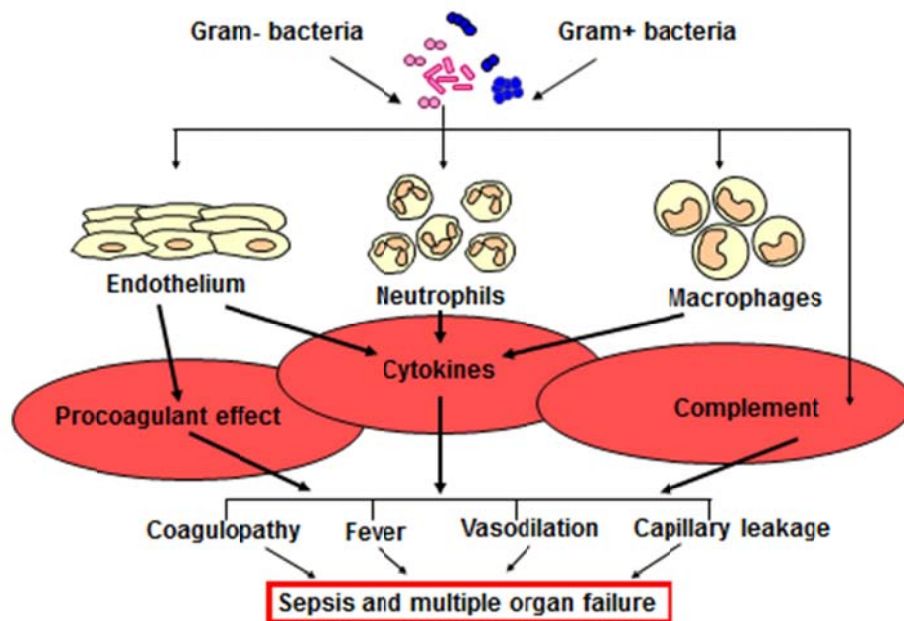


Figure 7. Schematic picture of the complex pathophysiology in sepsis leading to multiple organ failure.

1.3.3 Pathophysiology in severe sepsis and septic shock

The inflammatory response seen in infections is nowadays viewed as a balanced response between pro-inflammatory mediators (IL-1, IL-6, TNF- α) and the mediators produced in the compensatory anti-inflammatory response syndrome (CARS), by for example IL-1 receptor antagonists, IL-4 and IL-10, in order to balance up the pro-inflammatory responses [132, 133]. This regulatory anti-inflammatory response induces leukocyte anergy, reduction and apoptosis of lymphocytes, decreased pro-inflammatory cytokine response by monocytes to stimulation, loss of monocyte HLA-DR expression [134], decreased antigen-presentation by monocytes, as well as increased expression of the above immunosuppressive cytokines [25, 135]. A recent

study strengthens the hypothesis that the pro- and anti-inflammatory responses act in concert, by the induction of both pro- and anti-inflammatory genes in critically ill patients [136]. In best case scenarios, the innate responses of activated immune cells lead to a balanced response contributing to the elimination of pathogens and tissue recovery (figure 8A). In worst case scenarios, the responses lead to an imbalanced inflammation and tissue injury (mostly the pro-inflammatory response) or to a state of immune suppression (mostly the anti-inflammatory reactions) (figure 8B). The current understanding is that the inflammatory response in these patients is too strong in the initial stages, while at later stages patients have a reduced responsiveness of blood leukocytes to pathogens, and are left more fragile and highly susceptible to secondary infections [137, 138].

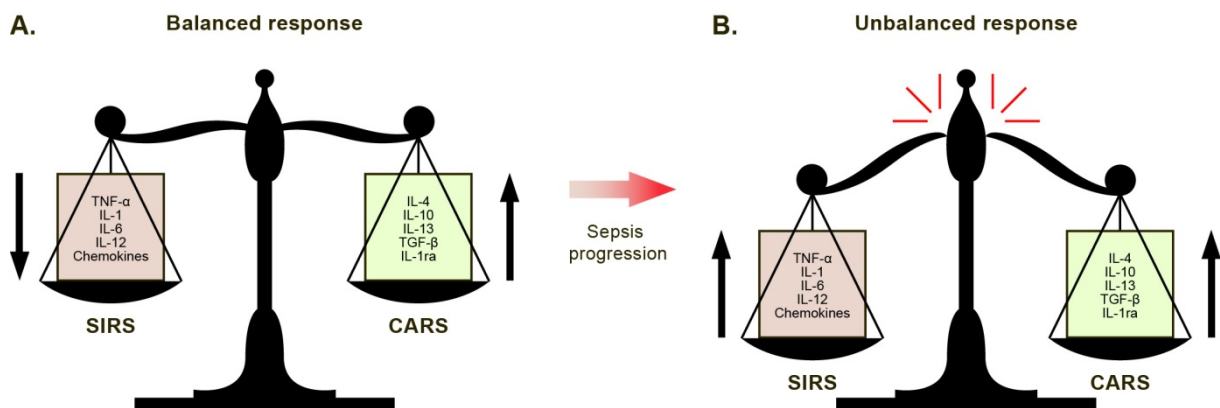


Figure 8. The host inflammatory response and the development of septic shock. In A, the inflammatory response is a balanced response between pro-inflammatory mediators in SIRS and anti-inflammatory mediators in CARS. SIRS mediators such as TNF- α and IL-1, IL-6 and IL-12 as well as chemokines activate the host immuno-inflammatory system causing tissue injury. The CARS mediators can deactivate leukocytes through the expression of IL-1 receptor antagonist (IL-1ra), IL-4, IL-10, IL-13 and TGF- β . SIRS and CARS are believed to work in concert. However, in the development of septic shock, B, the normally regulated expression of SIRS and CARS mediators is lost, resulting in an exaggerated and dysfunctional inflammatory response, with a potential deleterious ending. Modified from Buras et al. [139].

In addition to these responses, neural mechanisms are initiated, that can inhibit inflammation [140]. In the neuroinflammatory reflex, sensory input is relayed through the afferent vagus nerve to the brain stem, from which the efferent vagus nerve activates the splenic nerve in the celiac plexus, resulting in norepinephrine release in the spleen and acetylcholine secretion by a subset of CD4⁺ T cells. The acetylcholine release targets $\alpha 7$ -cholinergic receptors on macrophages, suppressing the release of pro-inflammatory cytokines [141].

The detrimental effects of a dysregulated inflammatory response and its contribution to systemic toxicity, as seen in severe sepsis/septic shock, were illustrated by the unfortunate outcome of the first phase 1 clinical trial of TGN1412, a novel superagonist anti-CD28 monoclonal antibody that directly stimulates T cells [142]. Within 90 minutes after receiving one single intravenous dose of the drug, all six healthy volunteers had a systemic inflammatory response characterized by a rapid induction of pro-inflammatory cytokines and accompanied by headache, myalgia, nausea, diarrhea, erythema, vasodilatation and

hypotension. Within 12 to 16 hours after infusion, the volunteers became critically ill, with pulmonary infiltrates and lung injury, renal failure, and disseminated intravascular coagulation. Severe and unexpected depletion of lymphocytes and monocytes occurred within 24 hours after infusion. All six patients survived with intensive care treatment including dialysis, high-dose cortisone, and an anti-IL-2 receptor antagonist antibody [142]. High inflammatory response with Th1 cytokines were observed in all patients within the first 4 hours following infusion, with a more prolonged course in the two patients with the worst outcome. These patients had particularly prolonged and high levels of IL-6, IL-4 and TNF- α , and one of them had less pronounced IL-10 level compared to the others. Another interestingly observation was that TGN1412, as illustrated above, elicited a much higher cytokine response in humans compared to that noted in non-human primates, clearly illustrating the difference in cytokine responses between human and primates and the obvious importance of careful research before conducting a clinical trial [143].

1.4 TREATMENT CORNERSTONES

Severe sepsis and septic shock are medical emergency conditions that should alert any staff member working in the health care system at any level, and be treated with the highest priority in patient care. “The golden hour”, the time period within which rapid treatment can make an outcome difference between life and death, is nowadays a well-known term to describe the fact that within an hour the clinician has a good chance to reduce mortality and also morbidity (“time is organ”). To influence outcome of disease, the appropriateness and speed with which sepsis therapy is administered should be similar to that of other emergencies like stroke, acute myocardial infarct and trauma. However, patients suffering from severe sepsis and septic shock can be difficult to identify early for staff working in an emergency department, and this area of knowledge has still a large potential of improvement. Evidence-based medicine and its implementation in daily practice are not easy tasks. Intensive education of the multidisciplinary teams working with these conditions is necessary to maximize chances of success. Decades of research trying to find the cure for the life-threatening conditions of severe sepsis and septic shock have still not come to the “final solution”, but care has improved using international guidelines provided by The Surviving Sepsis Campaign (SSC), an international consortium of professional societies involved in emergency medicine, critical care and treatment of infectious diseases. It was first established in 2004 in order to facilitate and improve management of sepsis [144-146].

Recent reports reveal that the implementation of the SSC care bundles is associated with an improved outcome [147, 148]. Principles of the initial management bundle are to provide cardiorespiratory resuscitation and to mitigate the immediate threats of uncontrolled infection. Resuscitation requires the use of intravenous fluids and vasopressors, with oxygen therapy and mechanical ventilation provided as necessary. The initial management of infection requires forming a probable diagnosis, obtaining cultures, and initiating appropriate and timely empirical antimicrobial therapy, as well as source control of the infection.

In summary and described in detail below, early identification of severe sepsis and septic shock and immediate adequate antibiotic treatment, as well as prompt, aggressive fluid resuscitation to maintain perfusion of tissues and oxygen delivery are the main cornerstones of treatment.

1.4.1 Initial screening and resuscitation

Early recognition and rapid commencement of treatment are perhaps the most important cornerstones for improved outcome in severe sepsis and septic shock. Biomarkers may help in the early detection and risk assessment of sepsis patients, and these are described in detail in a later section. Rivers et al. [149] reported on early goal-directed therapy (EGDT), an aggressive resuscitation program that significantly reduced mortality. Basically, EGDT is an active surveillance program of high risk septic patients, with aggressive treatment with fluids, inotropic drugs, blood transfusions as well as an invasive monitoring of central venous oxygenation. Studies of hospitals that implement EGDT protocol and surviving sepsis guidelines are clearly beneficial for the patients with an improved outcome. The absolute decrease in mortality has varied from 11-30 % [150-152].

1.4.2 Antimicrobial therapy

The introduction of antimicrobial therapy has made a huge difference in the surviving of infections. Unfortunately, we are now facing the rising global health problem of antimicrobial resistance, a looming problem for all infectious conditions and a tremendous threat to the management of severe sepsis and septic shock [153]. There are obvious differences in antimicrobial resistance between countries, and Scandinavia still has low rates, which will have an impact on epidemiological studies, further elaborated in paper I. Adequate, broad spectrum antibiotic therapy [36, 154-156] and timely [157-160] administration of antibiotics remain the most important treatments of severe infections. Each hour of delay in antibiotic treatment after onset of septic shock raises mortality with 7.6 % during the first hours, and time to initiation of effective therapy is the single strongest predictor of outcome [158]. The patient's previous medical history, recent use of antibiotics, the setting in which the infection developed, local microbial-susceptibility patterns, underlying diseases etc will often influence the choice of empirical antimicrobial therapy. The risk of inappropriate antibiotics is highest among patients with hospital acquired infections, patients previously treated with antibiotics and bacteremic patients with the highest Acute Physiology and Chronic Health Evaluation (APACHE) II scores.

Obtainment of appropriate cultures before antimicrobial therapy is recommended as long as it does not cause a significant delay (>45 min), with two or more blood cultures recommended [161]. Upcoming new methodologies using non-culture-based diagnostic methods (polymerase chain reaction, mass spectroscopy, MALDI-TOF, microarrays) may in the future perhaps not replace but help out in the early diagnostics of sepsis [162, 163]. New approaches to identify the conditions may help to narrow antibiotic therapy so as to reduce the risk of antibiotic resistance.

The choice of antibiotics and their administration can't wait for isolation and identification of the causative organism and determination of the organism's sensitivity to various antibiotics. Intravenous antibiotic therapy should be started as early as possible and should cover all likely pathogens. Thus, the antimicrobial therapy must almost always be broad and empiric. These principles underlie the observation that combination antimicrobial therapy may be superior to mono-therapy [164]. To prevent the emergence of resistant organisms, minimize the risk of drug toxicity, and reduce costs, a de-escalation of initial broad spectrum therapy to more specific therapy must take place as soon as possible [165].

1.4.3 Source control and supportive treatment

Identification of the focus of infection is a major part in the management of sepsis. Early-detection and adequate rapid antibiotic therapy alone may not be sufficient to treat the infection causing sepsis, in which case source control is also necessary to eradicate the infection [144, 166]. Drainage of an abscess, removal of an infected device and debridement of infected necrotic tissue are examples of source control that can make a difference in outcome of disease [166, 167].

The relative and absolute hypovolemia these patients are developing is of major importance to adjust for. Fluid therapy to reduce the development of organ dysfunction and multi organ failure is necessary. Initially, without any delay, should crystalloid fluid be rapidly administered ((500)-1000 ml/30 minutes) [146]. How the patient responds to fluid therapy (blood pressure >90 mm Hg, normalized diuresis (>0.5 ml/kg/hour), temperature and capillary refilling, repeated measurements of p-lactate) will guide the clinician to how much more fluid is necessary. As mentioned in the discussion of EGDT, the need of vasopressors and inotropic therapy is another supportive therapy as well as oxygen treatment [146].

1.4.3 Glucocorticoids

Glucocorticoids are widely used as anti-inflammatory agents in treatment of several chronic conditions. Their use in the treatment of severe infections is however greatly debated. Some studies have demonstrated a shorter time to shock reversal and improved survival [168-170], but, on the other hand, the incidence of hyperglycemia and superinfections was higher in the treated patients [170]. The guidelines of today is to only treat patients with vasopressor refractory shock with low-dose and short-course corticosteroid therapy (200 to 300 mg per day for up to 7 days or until vasopressor support is no longer required) supported by a meta-analysis study [171]. However, in patients with a chronic adrenal insufficiency (Addison's disease) and for patients that are already on corticosteroid therapy (>5mg prednisolone daily) the recommendation is to double the steroid dose when dealing with an infection with fever above 38 degrees, or triple the dose if fever above 39 degrees.

1.4.4 Adjunctive therapy?

Many attempts have been made during the past decades trying to target the activity of the dysregulated inflammatory response seen in severe sepsis and septic shock. Numerous studies have investigated new adjunctive therapies, some of which have been successful in animal models of sepsis, but translation to patients with sepsis has proved disappointing so far. Anti-inflammatory interventions specifically directed against early released cytokines, in particular IL-1, IL-1 receptor or TNF- α , all appeared promising in animal studies, but did not translate well into human clinical trials [172, 173]. Recombinant activated protein C, a promising molecule with anti-inflammatory, anti-thrombotic and profibrinolytic properties, was shown to reduce sepsis mortality [174], however, the side effects of bleedings [175] and the failure to reproduce the initial positive results has made the manufacturer withdraw it from the market [35].

1.4.5 Intravenous immunoglobulin therapy in sepsis?

Polyspecific intravenous immunoglobulin G (IVIG) has been suggested as an adjunctive therapy in severe infections. IVIG is pooled from thousands of donors and exhibits high polyspecificity, ensuring coverage of different superantigens and bacterial serotypes. However, clinical data have been difficult to evaluate. In sepsis overall, results of trials on IVIG as adjunctive therapy have been conflicting, and IVIG treatment is still controversial. The data available today is not sufficient to recommend the use of polyclonal IVIG for the entire group of sepsis, summarized in the recent Cochrane article [176]. The authors concluded that polyclonal IVIG reduced mortality among adults with sepsis but this benefit was not seen in trials with low risk of bias. The authors also concluded that in neonates with sepsis, there is sufficient evidence that standard polyclonal IVIG, as adjunctive therapy, does not reduce mortality based on the inclusion of the large polyclonal IVIG trial on neonates [177]. However, previous studies indicate that certain subgroups of sepsis such as STSS may benefit from IVIG (table 3) [178, 179]. The rationale for this beneficial effect is that a known risk factor for developing invasive GAS infection is a lack of antibodies to M protein and superantigens, discussed in previous sections [52, 114]. In the prematurely ended RCT [178], the trend to improved survival of IVIG in STSS was supported by the significant improvement in organ function assessed by Sepsis-related Organ Failure Assessment (SOFA) score.

Table 3. IVIG as adjunctive therapy in STSS

Study design	Mortality (%)		p value
	Controls	IVIG	
Observational cohort (cases 21; controls 32) ^a	14/21 (67%)	11/32 (34%)	0.02
Multicenter, placebo-controlled trial (cases 10; controls 11) ^b	4/11 (36%)	1/10 (10%)	0.2

^a Kaul et al. [179], ^b Darenberg et al. [178]

The molecular basis and mechanisms of action of IVIG are summarized in figure 9. Firstly, IVIG facilitates opsonization by marking the pathogen for ingestion and destruction by phagocytes. In patients with severe invasive GAS infection, increased levels of opsonizing anti-antibodies have been found in plasma post IVIG-treatments compared to pre-treatment samples [180]. Thus, increased bacterial clearance through opsonizing antibodies against GAS is one mechanistic action of IVIG contributing to clinical efficacy of these infections. Secondly, toxins released can be directly neutralized, since IVIG contains high levels of superantigen neutralizing antibodies, potently inhibiting the proliferative and cytokine-inducing capacity of streptococcal superantigens *in vitro* at physiological concentrations of IVIG [181, 182]. Thirdly, IVIG has anti-inflammatory effects through immunomodulatory activities believed to include Fc (fragment crystallizable) receptor-interactions, soluble immune components and inducing regulatory cytokines [183, 184]. The Fab (fragment antigen binding) portion of the antibody binds to the antigen, whereas the Fc portion of the antibody binds to an Fc receptor on the phagocyte, facilitating phagocytosis. The receptor-opsonin complex can also create byproducts like C3b and C4b which are important components of the complement system. These components are deposited on the cell surface of the pathogen and aid in its destruction.

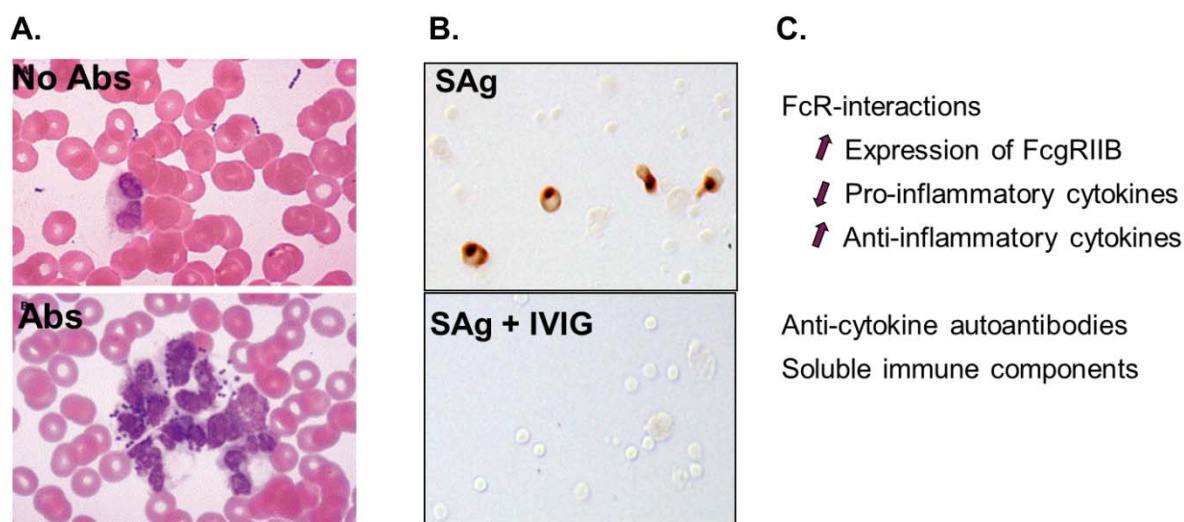


Figure 9. Mechanistic actions of IVIG. *A*, IVIG facilitates opsonization. *B*, toxin-neutralization is visualized; IVIG contains high levels of superantigen neutralizing antibodies. *C*, IVIG has anti-inflammatory effects through immunomodulatory activities believed to include Fc-receptor interactions, soluble immune components and inducing regulatory cytokines.

1.5 BIOMARKERS AND MEDIATORS IN SEPSIS

The pathways of sepsis pathophysiology involve extremely complex chains of events, including pro- and anti-inflammatory processes, humoral and cellular reactions and circulatory abnormalities. Early identification and risk assessment is highly important in management of patients with these severe conditions, increasing the possibility of starting timely, specific and correct treatment for reducing sepsis-associated morbidity and mortality [149, 185]. In this aspect of early detection, biomarkers work as diagnostic tools, defined as “. . . a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [186], or perhaps more easy to understand: “quantifiable measurement of biological homeostasis that defines what is ‘normal,’ therefore providing a frame of reference for predicting or detecting what is ‘abnormal’” [187]. The additional information provided by a biomarker may play a key role and provide insight into the pathogenesis and prognosis of a disease course and also aid in a clinical therapeutic decision, for example, by indicating the absence or presence or severity of sepsis, and differentiate systemic sepsis from a local infection. They may also predict complications and the development of organ dysfunction beyond what is provided by clinical examination and routine data [188]. A biomarker may also aid in risk stratification, finding subgroups of patients where the potential for benefit with a given therapeutic intervention is greater.

In this regard, more than 100 distinct molecules have been identified and evaluated for the diagnosis and identification of patients at risk for a poor outcome [189-191]. Most of them are still at an experimental state, but some are now being used in daily practice. For example, C-reactive protein (CRP), produced by hepatocytes upon IL-6 stimulation, is used since many years in detecting patients with infections, but its specificity has been a matter of debate [192-194]. Another marker of infection is procalcitonin (PCT), proposed as a more specific and better prognostic marker than CRP [195, 196]. Nowadays, PCT is used to direct the duration of antibiotic use [197, 198]. It remains difficult to differentiate sepsis from other non-

infectious causes of systemic inflammatory response syndrome, and there is a continuous search for better biomarkers of sepsis. Future potential biomarkers of particular relevance to this thesis are described in more detail below.

1.5.1 Triggering Receptor Expressed on Myeloid cells-1

The triggering receptor expressed on myeloid cells-1 (TREM-1), belonging to the immunoglobulin superfamily, has recently attracted attention as a diagnostic and prognostic biomarker for sepsis [199, 200]. Bacterial products can induce expression of TREM-1 in both monocytes and neutrophils and activation of this receptor and its soluble part (sTREM-1) during infection results in enhanced production of pro-inflammatory cytokines [201, 202]. Modulation of TREM-1 has been shown to downregulate the inflammatory response and improve disease outcome in murine models of sepsis [203], suggesting that anti-TREM-1 interventions may be an efficient therapeutic strategy for the treatment of sepsis. However, Lagler et al. [204] reported that TREM-1 confers protection and is required for the control of pneumococcal pneumonia infection, suggesting that the beneficial effect of TREM-1 modulation is largely dependent of the type of infection. In contrast, in a recent paper [205], a significant correlation between the levels of sTREM-1 in the plasma of GAS-infected septic patients and disease severity was seen, which also was confirmed in an experimental murine model of GAS sepsis. The results underscore the potential value of TREM-1 as a surrogate marker for monitoring patients with a specific sepsis, namely the streptococcal sepsis, as well as being a potential therapeutic agent for the treatment of invasive streptococcal infection.

1.5.2 Soluble Urokinase Plasminogen Activator Receptor

Another biomarker that could potentially indicate severity of disease is Urokinase Plasminogen Activator Receptor, a molecule embedded in the cell membranes of leukocytes. Its soluble counterpart, suPAR, is released during inflammation [206] and is reported as a marker of severity and predicts poor outcome in a variety of diseases ranging from certain types of cancer to infectious diseases [206-208]. Recent studies suggest that suPAR may predict mortality in critically ill patients [209, 210], especially in patients with pneumococcal infections [211, 212]. Both uPAR and its ligand, urokinase plasminogen activator (uPA) promote the migration and adhesion of leukocytes by binding to β -integrins [206]. uPAR may play a role in neutrophil recruitment and phagocytosis during infection, as indicated by studies of uPAR-deficient mice [213-215]. We sought to develop a prediction rule that aimed to improve prognostication with four levels of risk in sepsis based on APACHE II score and serum suPAR. The SuPAR levels were effectively combined with the APACHE II score to stratify ICU patients for risks of morbidity and mortality [216].

1.5.3 Resistin

Discovered in 2001 [217, 218], resistin is a 108-amino acid, 12.5 kDa peptide hormone of the cysteine-rich peptides called resistin-like molecules (RELM). Resistin has been shown to mediate insulin resistance in mice [219], of interest as acute infections are known to lead to an immediate insulin resistance and are followed by impaired control of blood glucose levels. The amino acid homology between human and murine resistin is only 59 %, which could explain the inconclusive findings regarding the potential role of resistin in human insulin regulation [220]. In humans, resistin has been identified as a potent pro-inflammatory molecule associated with acute and chronic inflammatory conditions [221-223].

Adipocytes are the main source of resistin in mice. In man, there are different reports of cellular origin of resistin, some papers suggests adipocytes [220], some report of monocytes as the origin [221, 224, 225]. Also, resistin has been detected in placental tissue [226] and pancreatic islet cells [227]. We report in **paper III** of a novel producer of resistin in human, namely the neutrophils. Resistin has been shown to stimulate synthesis and secretion of the pro-inflammatory cytokines TNF- α and IL-12 in macrophages through NF- κ B dependent pathway [228]. In PBMCs and in adipocytes, resistin induces TNF- α and IL-6 release [220, 223], where in the latter the c-Jun N-terminal kinase (JNK), or stress-activated protein kinase, signaling pathway is involved. However, no receptor for resistin has yet been identified. In PBMCs, LPS as well as IL-1, IL-6 and TNF- α , increase expression of resistin mRNA [229]. In addition, resistin up-regulates vascular cell adhesion molecule (VCAM)-1 on endothelial cells [230]. *In vitro* assays also show that intracellular adhesion molecule (ICAM)-1, important in the attachment of cells to a site of infection, is up-regulated on monocytes in response to resistin [231].

There are numerous reports on resistin and its role in inflammation. As described above, resistin induces production of several pro-inflammatory cytokines and there is a clear increase in resistin in patients suffering from rheumatoid arthritis, observed to accumulate in inflamed joints [232-234]. To date, there are few studies of the role of resistin in severe infection. Our group conducted a study to assess resistin levels during acute phase of infection in patients with varying severity of sepsis. The study revealed that resistin was strongly elevated during sepsis and was a significant marker of severity of sepsis (measured with SOFA and APACHE II scores) with increasing levels in severe sepsis and septic shock [231]. The levels of resistin remained elevated for a prolonged period during the acute phase of infection compared to other cytokines (figure 10).

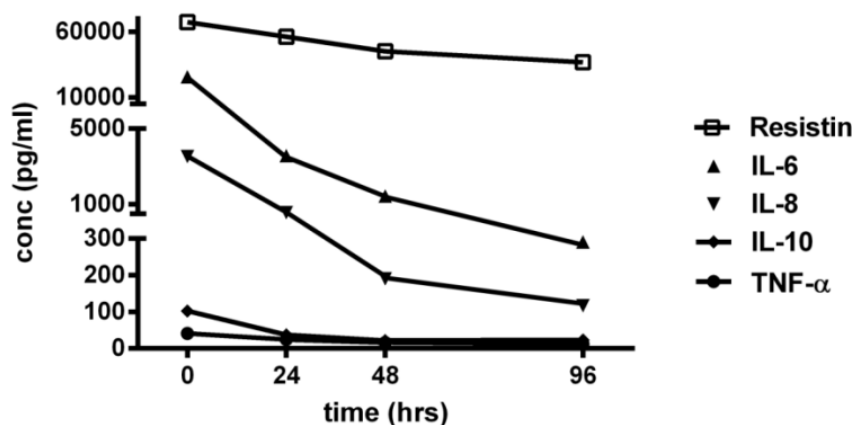


Figure 10. Resistin and cytokine kinetics in patients with severe sepsis and septic shock from a previous sepsis study in our group. Values are means. Modified figure from Sundén-Cullberg et al. [231].

1.5.4 Heparin-binding protein

Heparin-binding protein (HBP), also referred to as azurocidin or cationic antimicrobial protein of 37 kD (CAP37), is an inactive serine protease stored within both azurophilic granules and secretory vesicles in the neutrophils [99]. Identified in 1984 by Shafer et al. [235], it has been associated with many functions including antimicrobial- and immunostimulatory activity [236, 237]. It is a multifunctional granule-associated protein that is rapidly mobilized from migrating polymorphonuclear leukocytes. After secretion from the migrating neutrophils, HBP acts by binding to endothelium and thereafter presented to leukocytes in parallel working as a chemoattractant, activating monocytes [102, 238-240]. The antimicrobial effect is believed to be due to enhancement of antimicrobial effectiveness in monocytes [241], alternatively by direct opsonization of bacteria [242]. Impairment of the endothelium leading to plasma leakage and edema formation is a characteristic feature of the inflammatory process. It is known that migration of neutrophils is followed by efflux of plasma from the vasculature and that neutrophils trigger permeability changes themselves [243].

The role of HBP in infections is becoming more evident. HBP was found to be elevated in cerebrospinal fluid in patients with acute bacterial meningitis compared to other central nervous infections [244], suggesting HBP to be a useful diagnostic marker in meningitis. HBP is also involved in the pathophysiology of soft tissue infection. HBP was shown to be present in skin biopsies obtained from infected and erythematous areas of 12 patients suffering from erysipelas but not detectable in control biopsies from the non-infected leg [245]. This finding suggests that HBP could play an important role in the edema formation seen in streptococcal soft tissue infections. In another study, tissue biopsies from patients with necrotizing fasciitis and severe cellulitis caused by GAS revealed that the recruitment of neutrophils and monocytes/macrophages to the site of infection is accompanied by the release of HBP [102].

Of particular importance for this thesis, as an activator of macrophages and monocytes and a potent inducer of vascular leakage, HBP has been suggested as a central player in the pathophysiology of circulatory failure and life-threatening hypotension, thereby complicating severe infections [246, 247]. Importantly, it is a neutrophil-derived effector molecule associated with the severity of sepsis as well as being an early marker of circulatory failure in sepsis [248]. In this study by Linder et al., high HBP-levels were evident in ICU patients with severe sepsis and septic shock, and the levels correlated with disease and mortality, suggesting HBP to be useful in predicting outcome and treatment failure in ICU patients [248].

2 GENERAL AIMS

The aims of this thesis were two-armed: to study both *in vivo* and *in vitro* characteristics of severe sepsis and septic shock, and to define pathogenic mechanisms in neutrophil activation and degranulation with a specific emphasis on the role of HBP and resistin; recently identified markers of severity in sepsis.

Specific objectives:

- To document clinical presentation and outcome of severe sepsis and septic shock at the Intensive Care Unit of Karolinska University Hospital Huddinge, Sweden.
- To document clinical efficacy of polyspecific IVIG therapy in a prospective observational study of STSS, a subgroup of septic shock patients.
- To characterize resistin responses in patients with severe sepsis or septic shock with focus on its expression both systemically and locally.
- To explore whether neutrophil responses, in particular the release of the sepsis-associated factors HBP and resistin, differ depending on stimuli and how this relates to sepsis of different aetiology.

3 MATERIALS AND METHODS

Details about the methods used in this thesis are described in the original papers I-IV. Below follows a brief description of the various cohorts used in the separate studies, and some methodological considerations.

3.1 SUBJECTS

3.1.1 Paper I

This was a prospective observational study of a total of 101 patients with severe sepsis ($n=15$) and septic shock ($n=86$) (for definitions, see Bone et al. [10]) who were admitted to the ICU at Karolinska University Hospital Huddinge between 2005 and 2009. In addition to these patients, we also included sepsis patients enrolled in two previous prospective studies as a confirmatory cohort for comparison of mortality rates. These studies had similar or identical inclusion criteria and were conducted at the same study site between 1999 and 2001 ($n=54$; 22 severe sepsis and 32 septic shock) and between 2003 and 2005 ($n=50$; 9 severe sepsis and 41 septic shock). The study was originally designed for the inclusion of a control group of 28 non-infected severely ill patients admitted to the ICU. However, as it turned out, the inclusion of this “control” group was more difficult to evaluate than was predicted. As these patients are quite different in terms of illness eventually we did not include them at all in paper I. Slowly patient recruitment was due to several facts, most important having only one research nurse available. However the identification and enrollment of the patients could be done both day and night. Severity of disease was measured by APACHE II [249] at admittance and also by daily SOFA score until day 7 [250]. These scores and final diagnoses were determined retrospectively, on the basis of complete patient charts and laboratory tests. We retrospectively studied the timing of antibiotic administration and clinician evaluation in those patients that were admitted to the ICU through the ER ($n=43$).

3.1.2 Paper II

75 patients with STSS were identified in a national Swedish prospective surveillance study conducted between April 2002 and December 2004, where a total 746 patients with invasive clinical GAS blood isolates or isolates from other normally sterile site were collected from the microbiological laboratories and characterized using molecular techniques. The isolates were sent from all 29 Swedish microbiological laboratories to the Swedish Institute for Infectious Disease Control (recently incorporated into the Public Health Agency of Sweden). STSS was defined according to the definition proposed by “The Working Group on Severe Streptococcal Infections” [49]. For the 75 identified STSS patients within this group, questionnaires were sent out to the physicians asking for more detailed information regarding severity of disease, treatment (antibiotics, IVIG), surgery etc. The severity of disease was measured using the SAPS II [251]. Of the 75 questionnaires sent out to the attending physician, 69 came back properly filled out. Two patients were then excluded not fulfilling the STSS criteria, leaving 67 patients for further analysis.

3.1.3 Paper III

In this study of the role of resistin in severe infections, we analyzed patients with severe sepsis and septic shock enrolled in the two previous prospective studies described above (1999-2005), of which 92 patients had baseline values for resistin. For analyses of intracellular resistin in whole blood, five patients with septic shock were included from paper I. Samples from nine healthy volunteers were included as controls. For detailed analysis of STSS vs Gram-negative septic shock, we used patients with defined STSS (n=18) [178] or culture-confirmed Gram-negative septic shock (n=17) [231]. Serum/plasma samples were collected at several time points during the acute septic episode. Because the STSS patients were part of a placebo-controlled trial of IVIG, only samples at baseline before study drug administration were used from the patients that had received IVIG. Only patients that had received placebo (n=10) were included in the study of resistin kinetics. Ten snap-frozen tissue biopsies from patients with necrotizing fasciitis and STSS, as well as one biopsy from a patient with severe cellulitis, all caused by GAS, were used [113, 252]. As controls, snap-frozen tissue biopsies from five healthy individuals undergoing reconstructive surgery at Karolinska University Hospital were used.

3.1.4 Paper IV

Plasma samples from culture-positive severe sepsis and septic shock patients, including 20 and 28 patients with Gram-positive and Gram-negative bacterial infections, respectively, and a reference group of non-infected critically ill patients (n=28) enrolled at Karolinska University Hospital Huddinge [253] (**paper I**) were used in this report. The three cohorts were well matched with respect to age, gender and severity of infection based on APACHE II score at the day of inclusion. To further study specific streptococcal infections, plasma from patients with STSS caused by GAS (n=8) were provided from a sepsis study conducted at Lund University Hospital [248]. All plasma samples were collected during the acute septic episode. Immunohistochemically analysis were performed using snapfrozen tissue biopsies from patients with necrotizing fasciitis or severe cellulitis caused by GAS (n=9). No APACHE II score was available from the STSS-cohort. This material has been described previously (see above section).

3.2 LABORATORY METHODS OF HBP AND RESISTIN ANALYSES

All the specific methods used are described in detail in each paper. Some methodological comments are however needed, and therefore discussed below.

3.2.1 Analyses of patient samples or cell culture supernatants

Resistin was mostly analyzed by a commercial enzyme linked immune sorbent assay (ELISA). However for some multiplex analyses, Luminex was used. Importantly, a comparison of representative samples with both ELISA and Luminex analysis revealed essentially identical resistin levels. In addition, since our patient cohorts included both plasma and serum samples, resistin levels in serum and plasma samples collected from the same individual at the same time point from representative patients were analyzed with ELISA and no significant difference was seen in resistin levels. HBP was analyzed in plasma by ELISA as previously described [99]. IL-8 was measured in a multiplex Luminex analysis. Myeloperoxidase (MPO) levels were measured in cell culture supernatants by a commercial ELISA.

3.2.2 Bacterial strains and factors

In **paper III**, clinical isolates from blood cultures of patients with STSS or Gram-negative septic shock were used in the infection/stimulation assays, including GAS strains 5448 (M1T1 STSS isolate) and 08/04 (M1T1 STSS isolate), as well as *E. coli* blood isolates from two patients in the Gram-negative patient cohort. Supernatants were prepared from overnight cultures of the above-mentioned GAS strains. Such supernatants contain a mixture of secreted superantigens and other exotoxins. Supernatants were also prepared from cultures of the AP1 strain and its isogenic mutant MC25, which contains a truncated form of the M1 protein that lacks the transmembrane-spanning region, leading to M1 protein accumulation in the supernatant.

In **paper IV**, blood isolates, including GAS (n=4), *E. coli* (n=2) and *S. aureus* (n=2), collected from the septic shock patients in respective cohort described above in detail were used. The GAS strains were of serotypes T1 (n=2), T4 and T28. In addition, streptococcal strains, including group B (GBS; n=1), group C (GCS; n=2), group G (GGS, n=1), and *S. viridans*, isolated from patients with severe sepsis were used.

3.2.3 Analyses of tissue biopsies and primary human cells

To identify the amount and the cellular source of HBP and resistin, we relied much of our research on immunohistochemical stainings and computerized image analysis. This is a semi-quantitative method to study protein expression in cryo-preserved tissues, exploiting the principle of antibodies binding specifically to antigens in biological tissues. This method is widely used in research laboratories to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a tissue. High-resolution images can be taken at different magnifications using a digital camera connected to the microscope. The digital software is able to determine the percent positive area of the total cell area as well as the total mean intensity of the staining. The results are then presented as acquired computerized image analysis (ACIA) values which equal the percentage of positively stained area x the mean intensity of positive staining. Single and dual immunofluorescence stainings of both tissues and cells to detect co-expression of proteins were done by multicolor labeling and evaluated by a Leica confocal scanner coupled to a Leica microscope. Since this software can differentiate an extensive range of colors (up to 16.7 millions), it supports detailed assessment of different proteins. Quantification of the immunofluorescent stainings is usually performed manually. For a more specific quantification and measurements of the potential co-localization of HBP and Resistin in **paper IV**, we used a Nikon confocal microscope in combination with a software analysis called Imaris 3-D image analysis. This method is used to provide functionality for the visualization, segmentation and interpretation of 3D microscopy datasets to discover relationships that are otherwise hidden. To re-capture the full area of the cells, the Imaris surface function was used based on the alexa546 channel. To automatically locate the vesicles inside the cells, the Imaris cell function was applied based on size and intensity thresholds defined by the user. Measurement of co-localization was carried out by masking each respective vesicle type, creating new channels for each marker. The total number of co-localized pixels was measured, and thresholds were automatically computed by using orthogonal regression analysis of the image's scatterplots in combination with the Pearson's coefficient approach.

3.3 STATISTICAL ANALYSES

Descriptive data are presented as mean (SD) for continuous data, and medians with interquartile ranges (IQRs) for numerous data that did not follow Gaussian distribution. To test for normality, we used recommended D'Agostino and Pearson omnibus normality test. Comparisons between groups were made by the non-parametric Mann–Whitney *U* test or Kruskal Wallis with Dunns test when appropriate, or for categorical values, Fisher's exact test. The survival analysis was made by using Kaplan–Meier survival curve. The analysis of risk factors for death in the septic cohort was performed using a multivariate cox regression analysis performed in two steps. All factors with a univariate *p*-value of <0.1 were entered into a stepwise Cox regression model where the model selection was based on the Akaike Information Criteria (AIC) approach. Correlations between variables were determined by use of Pearson correlation test or, in the case of non-Gaussian distribution of the data, Spearman rank correlation coefficient. The GraphPad Prism version 4-6 (GraphPad Software, La Jolla) was used for all statistical analyses except the multivariate analysis where the R version 2.14.1 was used in **paper I** and STATISTICA version 12 (StatSoft Inc., Tulsa, USA) in **paper II**. A two-tailed *p*-value <0.05 was considered statistically significant.

3.4 ETHICAL CONSIDERATIONS

All four studies were conducted in accordance with the declaration of Helsinki and were approved by the local ethics committee of Karolinska University Hospital, the Regional Ethical Review Board at the Karolinska Institute, the University of Toronto, and Lund University Hospital. Written informed consent was obtained from the patients or their close relatives, and is archived by the authors.

4 RESULTS AND DISCUSSION

As previously described, the project was “two-armed” in a clinical and pathophysiological way. I will therefore here first focus on the two clinical papers and then describe the results of the more experimental papers III and IV.

4.1 MORTALITY IN SEVERE SEPSIS AND SEPTIC SHOCK (PAPER I)

Despite adequate antibiotics and modern intensive care, severe sepsis and septic shock are associated with high mortality rates. The progress towards new treatments for sepsis has been slow, in part explained by the fact that many sepsis studies are heterogeneous when it comes to comparing severity of disease, comorbidities and etiological aspects, thus making it very difficult to evaluate the effect of a certain treatment therapy or other procedures in the ICU. Not much is known of how much we can actually gain on new agents and therapies regarding sepsis mortality. We believe that a successful clinical trial evaluating an immunotherapeutic agent in sepsis requires well defined patient cohorts with respect to severity and microbiological aetiology. In light of this, we wanted to seek deeper knowledge into how the actual outcome in these conditions presents in modern time in our University Hospital. For this purpose, we conducted a prospective observational study of severe sepsis and septic shock (n=101) in a mixed medical and surgical ICU in Karolinska University Hospital, Huddinge.

In this study, we demonstrate low mortality rates, both short- and long-term. Of importance when analyzing baseline characteristics of our patients, is the fact that our cohort is comparable with cohorts of previously reported international studies in respect to severity of sepsis (defined by severity scores), age, and underlying conditions [28, 158, 170]. The heterogeneity of sepsis studies mentioned above is visualized by the fact that there was a dominance of abdominal infections in our study, with *E. coli* being the most prevalent pathogen (figure 11).

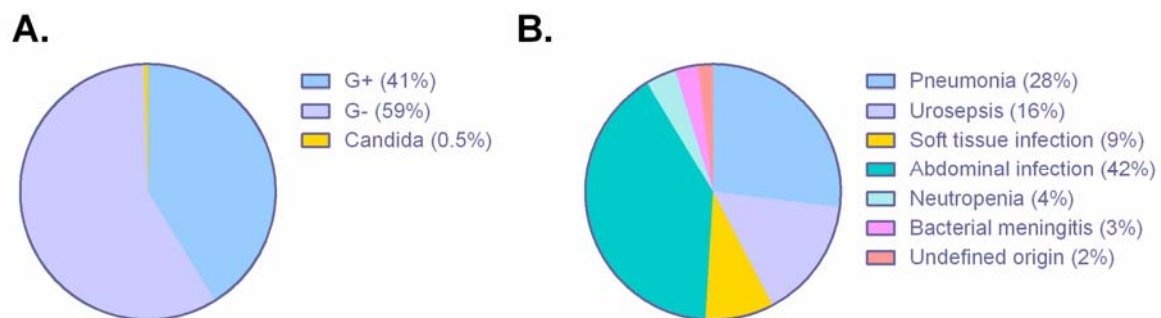


Figure 11. Culture findings and clinical manifestations. A, Of positive blood-cultures, the Gram-negative pathogens dominated and *E. coli* in particular, in line with B, where the most common conditions were the abdominal infections.

This finding is explained by the fact that upper gastrointestinal surgery is centralized to Karolinska University Hospital Huddinge. The abdominal dominance is in contrast with, for example, the study by Heffner et al, where *S. aureus* dominated [254]. This substantial heterogeneity must be taken into consideration when conducting new sepsis trials.

Our low mortality rates are discussed in detail below and may be explained by the combination of a well-functioning triage system that directs the patient to the right priority group, the lack of resistant isolates and short-time to adequate antibiotics together with early aggressive fluid resuscitation. Although the majority of the patients received adequate antibiotics from the beginning, the data suggested that women with community-acquired severe sepsis and septic shock had a delay in antibiotic treatment as compared to men; a concerning observation that warrants further studies.

4.1.1 Outcome

Mortality in severe sepsis and septic shock can roughly be divided into three parts. The almost immediate deaths (half of the patients) are often due to refractory shock and acute cardiovascular collapse. The second mortality (within a month) is due to multiple organ failure, where the therapy must be focused on specific organ support. Thirdly, later on during the clinical course, there is evidence that the patients die of a persistent low-grade inflammation [255]. In the study by Quartin et al., sepsis increased the risk of death for up to 5 years after the septic episode even after comorbidities are accounted for, in comparison to controls from the general populations. The risk of late death during the first year is associated with the severity of the septic episode [256].

In this study, the primary and secondary end points were short- and long-term mortality (day 28 and day 365), as well as hospital mortality. We reported on mortality rates of 19% (day 28), 29% (hospital) and 34% (1 year). Both short- and long-term mortality were due to a septic shock with multiple organ failure which in the late mortality cases were followed by cardiac heart failure, pneumonia, cardiac arrest, acute myocardial infarct, and liver failure.

When comparing mortality rates it is important to study reports with similar inclusion criteria and severity scores. The noted mortality rates in our study are lower than, for example, that reported in the study by Kumar et al. [158], with a reported hospital mortality rate of 56% in septic shock patients, and the study by Quenot et al. with a 28-day mortality of 42% [257]. The study by Ranieri et al. [35] reported of a 28-day mortality rate of 25.3%. In the European multicenter study by Sprung et al. with similar age and SOFA score as in our study, the 28-day mortality rate was 33% and the hospital mortality rate was 43% [170]. Comparing with other Scandinavian countries, the mortality rates in our study are in line with the Finnsepsis study for example [28]. An important note is the fact that our septic cohort includes 15% severe sepsis patients whereas all the others had septic shock. However, inclusion of only septic shock patients from our cohort resulted in even lower mortality rates; day 28 mortality of 17% and hospital mortality of 29%.

Originally we also included a non-infected critically ill cohort as to compare mortality and baseline characteristics, but this “control” group was so diverse in diagnosis that it proved impossible to use for this purpose. In retrospect, we should have included a more homogenous group of control patients, such as solely trauma patients, for better comparison. However, we noted a clear difference in terms of mortality; the 28-day and 1-year mortality were 41% and 48% respectively in this group. The patients in the control group that did not survive died almost immediately, as compared to the sepsis patients who, if they survived the immediate incident, they often died later on, most likely due to a persistent inflammation.

4.1.2 Factors influencing outcome

In the multivariate analysis in our study, risk factors for death were age, cardiac heart failure, immunosuppression, and SOFA score. Many are the factors that can potentially influence mortality rates in varying settings, not the least different health care systems and approaches to critical care [9]. Severe sepsis and septic shock are acute conditions that need urgent attention and care, and recent studies highlight a trend toward decreasing case fatality rates connected with earlier recognition and earlier treatment [17, 258]. A correct diagnosis and commencement of therapy can make a huge difference in outcome of these patients; saving organs and lives. Without this immediate process, the prognosis is poor. Triage is the first step in the process; by determining the priority of patients' treatments based on the severity of their condition the clinician can act appropriate (see figure 7). The triage system is based on two arms; one concerns and focuses on patient safety, and the other focuses on improving the processes in the ER (patient flows). To date, three different triage systems are applied in Sweden; METTS (Medical Emergency Triage and Treatment System), MTS (Manchester Triage Scale) and ADAPT (Adaptive process triage). The latter was introduced in 2007 in Karolinska University Hospital. Evaluating the different systems has been inconclusive [259]. This study was not conducted to evaluate the triage system but it seems lightly that triage is one component in the process managing sepsis patients that may improve outcome. ER physicians play a key role in determining the level of care required by admitted patients as well as initiating appropriate interventions. Symptoms that should alert any staff are visualized in table 4.

Pathological signs:

- Respiratory frequency >20, SaO₂ <90%
- Blood pressure systolic <90 mmHg, MAP <70 mmHg, heart rate >90, impaired peripheral circulation
- Impaired consciousness, disorientation, agitation
- Diuresis <0.5 ml/kg/hrs
- Body temperature >38°C or <36°C
- Lactate >2.0, BE <-5

Table 4. *Impaired vital signs of patients with suspicious sepsis. Immediately in the ER, the patients are to be sorted, triaged, into different caring processes.*

Many studies have shown that the prognosis of severe sepsis and septic shock can be improved by using internationally recommended guidelines [160]. In analogy with the treatment of acute myocardial infarction or acute trauma, the efficacy and speed of early management and adequate treatment in the initial hours after onset of illness are likely to influence outcome. EGDT (described in the introduction) in severe sepsis and septic shock patients has been shown to improve 1-year mortality rate compared to standard treatment [149]. In our study there was no adherence to a specific EGDT protocol, yet, the 1-year mortality rate matches this outcome. In addition, the severity of disease in our septic shock patients was higher (SOFA score 10.4 vs. 7) compared to the patients in the study of Puskarich et al. [260], in which they reported of a 1-year mortality rate of 49% in the non-treated arm, suggesting that our patients were more severely ill. That would if anything have a negative impact on survival.

At the time of the study the recommended national guidelines in Sweden regarding cortisone treatment was a low dose of corticosteroids for septic shock patients in the need of inotropic support. To date, it is restricted to those with persisting hypotension despite inotropic therapy. 73% of our septic shock patients received hydrocortisone, indicating under prescription by guidelines at the time, but over prescription by current standards. Over prescription likely had no effect on mortality.

The heterogeneity concept has been discussed previously, and there are few data related to the effects of different sources of infection on outcome. It has been suggested that abdominal infections may be more severe than respiratory infections [261, 262]. A recent study showed no differences in age, sex, severity score, or mortality rates between the two groups, but the development of septic shock, early coagulation, and acute renal failure was more common in patients with abdominal infections [263]. The fact that abdominal infections dominate in our study should, if anything, influence our mortality rates negatively, which they did not.

Adequate and prompt antibiotic treatment is crucial for survival [155, 156, 158]. In total, 93% of the patients in our cohort were given adequate antibiotics from the onset, which likely contributes at least in part to the low mortality rate. To date, Sweden has managed to contain antibiotic resistance with low rates of MRSA (<1%) and *E. coli*-producing ESBL (<5%), and first-line antibiotics still work – for example, *S. pneumoniae* is routinely treated with penicillin G or V [264-266]. In addition, we report that the majority of patients, where timing was obtained in the ER, received appropriate antibiotics within 2 h, which is shorter than in many other studies reporting 3 hours or longer [267, 268].

4.1.3 Impact of gender in the care of patients

The potential impact of gender in terms of incidence and outcome in sepsis is reported inconsistent: some studies found a higher risk in men [269], some in women [270], and some found no difference [39]. In this report, no difference in outcome according to gender was noted. However, we report, for the first time, that gender influences time to antibiotic treatment. We analyzed the subgroup of 43 patients admitted to the ICU through the ER, and a troubling trend of gender difference in time to seeing a clinician and receiving adequate antibiotics was observed. Considering the strong link between time to antibiotics and outcome of severe sepsis and septic shock, this is a clinically important finding. In an attempt to seek the underlying reason for this delay in treatment, the female and male cohorts were compared with respect to diagnosis, aetiology, and severity of infection but no differences were identified except the fact that the females were younger. However it should be noted that this subgroup analyses is based on a small patient cohort and the results need to be verified in larger studies. Analysis of for example sepsis-registers would allow for such a study of potential gender differences in the future.

4.1.4 Personalized medicine in sepsis

The study also highlight the fact that, based on the heterogeneity discussion, it is time to reconsider the designs of future sepsis trials, and the fact that with these low mortality rates new studies of treatments may be a failure. Since different microorganisms have specific mechanisms leading to organ failure and death, the need of personalized medicine and the search for targeted patient groups is urgent. Before even initiating a trial of a new agent or intervention, one of the goals should be to gain access to the host biological and genetic

information, focusing on the conditions with a reasonably high mortality. The therapeutic compound to be tested must have a strong pathophysiological role, with robust mechanistic *in vitro* data. In addition, we need to be sure that there is a biological role of the targeted factor in the pathophysiology of the particular disease being studied. In the ideal world, when we wish to study interventions directed against specific mediators, we should identify patients in whom we can be certain that the target mediator is present at significant concentrations at the time at which the treatment is to be administered. As sophisticated diagnostic strategies with new tools to rapidly obtain a microbiological diagnosis are becoming available on the market, this could soon be a reality.

4.2 IMMUNOGLOBULIN THERAPY IN STSS (PAPER II)

The aetiology of severe sepsis and septic shock is commonly heterogeneous as previously discussed. For example, a majority of the patients in **paper I** suffered from abdominal origin of *E.coli* bacteremia. Subgroups of sepsis patients are to be treated differently depending on clinical presentation and aetiology. The site of infection and the bacteria causing the condition should therefore be taken into consideration individually in respect to many things, for example antimicrobial therapy and source control, and the addition of adjunctive therapy in targeted patient groups. Our group has previously focused much research on streptococcal infections, in particular the severe invasive GAS manifestations like STSS and NF. Despite adequate and immediate antibiotic treatment and surgery when needed, the mortality rates in STSS commonly exceeds 50% [51, 271, 272], a mortality rate higher than that of meningococcal septicemia for example. The awareness of STSS among health-care professionals is still low, the initial clinical picture may be vague with only fever and influenza-like symptoms, and STSS may present anywhere within the health-care system, with a fulminant disease progression that, once seen, is never forgotten. IVIG has been suggested as an adjunctive therapy to improve outcome in severe infections. In the STSS subgroup of septic shock patients studies, including an observational cohort study as well as small placebo-controlled trial, have reported decreased mortality rates in patients treated with IVIG [178, 179]. Even though these reports, together with the detailed mechanistic action, supports the use of IVIG as adjunctive therapy in STSS, the level of clinical evidence is low and there is a need for additional studies on this topic.

To further document on the clinical efficacy of IVIG in STSS, we conducted a prospective observational study. The study originated from a national Swedish prospective surveillance study of a total 746 patients with invasive GAS infection, including 75 STSS patients identified between April 2002 and December 2004 [90]. The analytic plan included a subgroup analysis of the presence of NF or not, since there is only anecdotal data available on IVIG therapy in NF [273], along with the analysis of other factors expected to contribute to improved survival like clindamycin and surgery.

In this study (paper II), a significant reduced mortality rate was noted in the IVIG treated group. In addition, the results give further support for the combination treatment with penicillin and clindamycin.

4.2.1 Clinical characteristics and treatment

For the 75 patients with STSS, extended clinical and therapeutic information was collected through a new questionnaire. 69 came back properly filled out. Two patients were then

excluded not fulfilling the STSS criteria, leaving 67 patients for further analysis (figure 12). Of these 67 patients, 23 (34%) received IVIG and 44 (66%) did not. Baseline characteristics between the two groups of IVIG-treated and non-IVIG treated patients revealed that both groups were well matched with respect to gender, age and severity of disease measured with SAPS II score. The IVIG patients were slightly younger (60 vs 65 years) and had a higher degree of NF.

A β -lactam antibiotic therapy was given to all patients. In addition, most patients also received clindamycin (78%), with a slight difference (not statistically significant) between the both cohorts, 91% in the IVIG group vs 70% in the non-IVIG group. Most of the patients received antibiotic treatment against GAS quite promptly, as visualized by time to adequate antibiotics (registered in 42 (63%) of the patients) with a median time of 1.3 hours (range 0.1-4.2). No difference between the groups was observed. Surgical procedures were undertaken to a higher extent in the IVIG-group, in line with the fact that this group had a more frequent diagnosis of NF for which surgery is the recommended therapeutic strategy.

Starting during the first day of onset of illness, the IVIG therapy administered was either Endobulin[®], Gammagard[®] or Octagam[®]. The dosage of IVIG was 0.5 g/kg in all except one case who received 1.0 g/kg, with a variation of duration of one to six days (8 cases one day, 5 cases 2 days, 2 cases 3 days, 4 cases 4 days, 3 cases 5 days and 1 case for 6 days). An adverse event consisting of a general rash was registered in only one patient in our study.

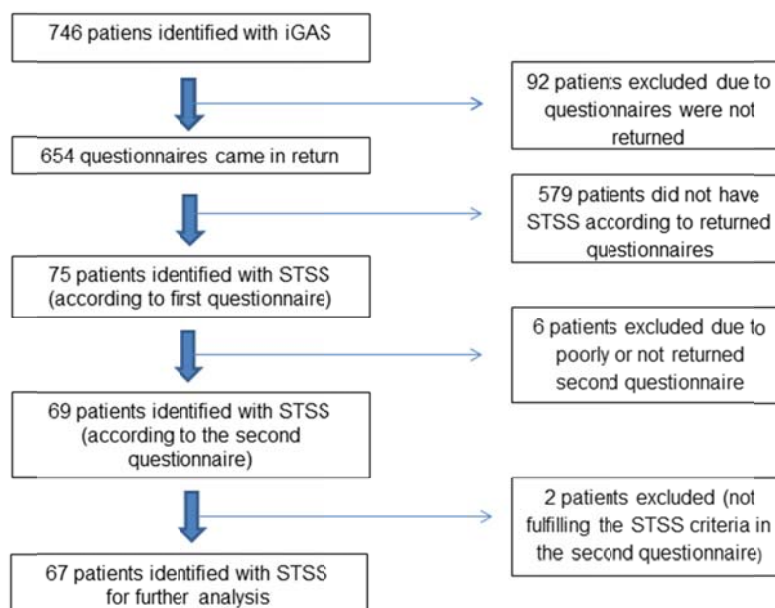


Figure 12. Flow chart identifying 67 STSS patients.

4.2.2 Outcome

The primary study endpoint was 28-day survival. The results demonstrate a significant difference in outcome between the IVIG group and the non-IVIG group (87% versus 50%, $p < 0.01$). Already after 7 days this difference was obvious (91% vs 59% ($p < 0.0001$)).

respectively). To address the noted difference in age between the cohorts, a post-hoc analysis was performed that analyzed the influence of age on the effect of IVIG. A cut-off of 80 was chosen based on two facts; one that patients in this age group receives the highest score in the age parameters of SAPS II and the other that a peak in incidence of invasive GAS infections indicating the increased susceptibility is seen in this group [274]. Under the age of 80 years, IVIG improved survival significantly ($p=0.039$). Although no significant effect was observed in the eleven patients in the STSS cohort above 80 years, data suggest improved survival by IVIG also among these patients, since the two patients given IVIG both survived in comparison with only two of the nine patients in the non-IVIG group.

4.2.3 Factors influencing outcome

According to the analytical plan the effect on survival of IVIG was first analyzed in all patients followed by evaluation of the two subgroups with and without NF. Similarly, clindamycin therapy, surgery and SAPS II score were analyzed in the univariate analyses as they represent potential confounders due to their expected association with outcome [54, 251, 275]. Finally, all these factors were analyzed in a multivariate analysis. The univariate analyses showed as expected that low SAPS II score, clindamycin treatment, IVIG therapy and surgery had significant effects on survival in STSS. When the subgroups with and without NF were analyzed separately, differences of the same magnitude were found, thus, the results indicate a similar effect in patients with NF as in those without.

In theory, the presence of NF would alert the clinician to a more rapid diagnosis of potential STSS for which aggressive therapy such as IVIG is proposed; thereby explaining the higher incidence of NF in the IVIG cohort. Considering the severity of NF, addition of this complicating factor should have a negative impact on outcome making the survival results even more impressive. As noted above, NF might contribute to a more prompt and aggressive therapeutic approach with clindamycin, IVIG and surgery. This was controlled for by subgroup analyses of SAPS II, clindamycin, surgery and IVIG in NF versus non-NF patients where similar effects were seen. When clindamycin, SAPS II score, surgery and IVIG therapy were analyzed together in the multivariate analysis, low SAPS II score and clindamycin were significant factors for survival. The effect of IVIG was almost significant. As surgery did not contribute to outcome it was excluded in the final analysis. In this final analysis, IVIG demonstrated a significant effect on survival with an OR of 5.6.

A change of treatment recommendations with the inclusion of IVIG as adjunctive therapy in STSS was introduced in Sweden in the later period of the study. Although the percentage of IVIG therapy in the total patient group increased from 27% to 56% in the period following the recommendation, there is a substantial portion of STSS that did not get the recommended treatment. This is most certainly a consequence of the low evidence level of the published clinical studies, which likely results in a tendency that younger, previously healthy individuals with a complicated manifestation, i.e. STSS in combination with NF, are more prone to receive IVIG. Similarly, Valiquette et al. noted a substantial variability between Canadian physicians in the use of IVIG for STSS [276], which underscores the need for further clinical data on this topic. We demonstrate a significant reduction in mortality in patients treated with IVIG as adjunctive therapy in STSS, both with uni- and multivariate analysis. Our survival rates are in agreement to that reported in the prematurely ended multicenter placebo controlled IVIG trial (90% vs 64% survival in the IVIG vs placebo group) [178]. An obvious strength of our observational study compared to Kaul et al. [179], is the usage of prospectively identified STSS patients including both treated and non-treated cases. In the retrospective pediatric study by Shah et al. [277], the

authors concluded that IVIG had no effect on survival with a mortality of 4.5% in both the IVIG and non-IVIG groups. Major concerns have been raised regarding this study, most importantly the fact that it was markedly underpowered considering the low mortality rate [278]. In addition the inclusion criteria used in the study did not follow the STSS definition criteria and were likely to result in inclusion of patients with a milder disease, as also evident by the low mortality rate and the fact that many of the cases did not require intensive care.

The survival analysis revealed an important finding that clindamycin had a significant effect on survival with an OR of 7.8. The overall recommended antibiotic regimen has been penicillin in combination with the protein-synthesis inhibitor clindamycin since the report of Stevens et al. [57]. However, the clinical data to support this is limited to two reports showing a beneficial effect in invasive GAS infections, particularly in those with NF/deep tissue infections [54, 55]. In our paper, among STSS patients without NF, clindamycin remained significantly associated with improved survival (OR 4.6). As the group of STSS patients with NF included only one patient who died, and this case had not been treated with clindamycin, the analysis could not be conducted in this specific group.

As mentioned, a randomized controlled trial (RCT) was previously conducted but had to be prematurely ended due to slow patient recruitment [178]. In the case of diseases that are rare, an RCT can be technically challenging to achieve and the quality is impacted by the low number of patients recruited at respective site. In these cases, observational studies like ours become even more important. When analyzing the differences in treatment-effect between an RCT and an observational study, many studies have observed a somewhat higher effect in the observational studies [279-281]. However, McKee et al. did not find it evident that observational studies gave systematically a higher treatment effect than randomized trials [282]. A well-designed prospective observational study can often match the results in a high-quality RCT, a concept highlighted in two studies in *New England Journal of Medicine* [283, 284]. Observational studies should be based on their methodological qualities, not the type of study design, as there is evidence that the scientific quality of a separate study have more impact on reliability than a certain study design. However as evident in our study, skewing between cohorts due to clinical practice may occur which could have been avoided in a randomized trial by use of stratification.

4.3 NEW BIOLOGICAL MARKERS/MEDIATORS (PAPER III AND IV)

Understanding the complexity of the pathophysiology in severe sepsis and septic shock is a challenge to every clinician and researcher in the field. Numerous are the inflammatory markers and mediators systemically released during the sepsis event and the responses are more prolonged and diverse than was previously thought [285]. Often mentioned in the context of sepsis are for example TNF- α , IL-1 β , IL-6, IL-8 and IL-10; commonly secreted rapidly after bacterial infection. An important clinical observation in the management of severe sepsis is that patients often succumb to death long after initial infection, at a time point when the early release of cytokines previously mentioned has long since passed. This observation has focused recent research on late mediators of inflammation, such as macrophage migration inhibitory factor (MIF) [286] and the nuclear protein HMGB-1 [287, 288]. The up-regulation of cytokines that persist for a prolonged time has been suggested to influence long-term outcomes of sepsis [289]. In our group, we have previously described the recently discovered protein resistin as being a marker of severity of sepsis with a sustained secretion profile as compared to early cytokines [231]. The highest systemic resistin levels were noted in patients with septic shock, and we also noted a trend of the confirmed Gram-positive infections to have higher levels of

resistin than patients with confirmed Gram-negative infections. In **paper III**, we explored further the resistin release with respect to cellular source in sepsis and relation to severity of infection systemically and locally in patients infected with the Gram-positive bacterium GAS. A major finding was the identification of neutrophils as a novel and dominant source of resistin in sepsis patients. In light of these findings, HBP (also neutrophil derived) was of interest to study, since it serves as a potent inducer of vascular leakage and hypotension seen in septic shock, and is found to be released upon GAS M1-protein neutrophil activation [106, 246, 247]. In **paper IV**, we further explored whether these neutrophil responses, in particular HBP and resistin-release, differ depending on stimuli and how this relates to sepsis of different aetiology (section 4.4).

4.3.1 Resistin in circulation and in tissue of infection (paper III)

In paper III we determined the resistin levels in circulation, with the use of well-defined patient cohorts comparing confirmed Gram-negative septic shock patients with patients with STSS (see materials and methods, section 3). These two cohorts were well matched in terms of gender, age and SOFA scores. The STSS cohort had equally high serum resistin levels at enrollment as compared to those with Gram-negative septic shock. However, markedly elevated resistin levels were noted at later time points, and the resistin levels measured in day 28 samples from the STSS patients were interestingly almost 2-fold higher than the day 14 samples (this was the latest time point available) from the Gram-negative cohort (figure 13).

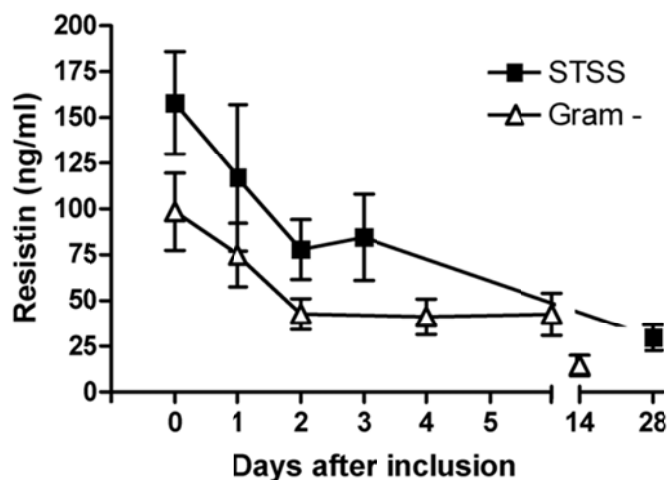


Figure 13. Prolonged levels of resistin in STSS patients (n=10), and Gram-negative septic shock patients (n=11) during the acute sepsis episode.

It is tempting to speculate that the prolonged elevation of systemic resistin, with known pro-inflammatory activity, could indeed play a central role in maintaining an inflammatory response believed to contribute to long-term mortality in sepsis. Our current material was too limited to allow for such conclusions, but it is noteworthy that four of the six STSS patients with elevated resistin levels 28 days after onset of the septic episode, demonstrated elevated IL-8 responses at this late time point. For future studies it would be interesting to compare resistin levels in STSS vs Gram negative patients enrolled in the same study.

To further explore resistin responses in clinical settings, studies of biopsies at the local tissue site of infection in patients with severe GAS infections were performed. In agreement with the elevated levels of resistin observed in serum, high levels of resistin were found in streptococcal tissue infections and, most importantly, a novel source of resistin was evident, described in the next section.

4.3.2 Source of resistin in bacterial septic shock

The cellular source of resistin was previously believed to be mainly monocytes, macrophages and adipocytes. The tissue biopsies of patients with severe GAS infections were immunohistochemically analyzed and resistin was found in all infected biopsies with a distinct cytoplasmic staining with a large percentage positive cells. Confocal microscopy performed with resistin and specific cellmarkers identified resistin-positive tissue macrophages, however, neutrophils represented the dominant source at the inflamed tissue site (figure 14). On average, 87% of the resistin-positive cell population were neutrophils as compared with 34% of the macrophages. This finding is in line with a later report by Bostrom et al. in which neutrophils were identified as an important source of resistin in patients with arthritis [290].

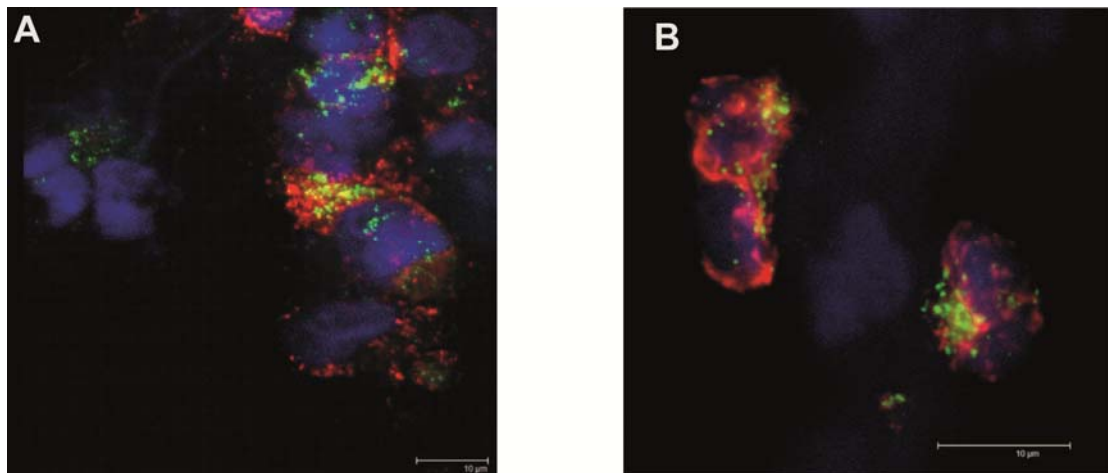


Figure 14. Cellular source of resistin at the tissue site. A: Resistin in green and CD68-positive macrophages in red. B: Resistin in green and neutrophil elastase-positive PMNs in red. Blue indicates cell nuclei. Scale bar indicates 10µm.

To assess whether neutrophils are also the dominant source of resistin in circulation, we performed fluorescence-activated cell sorting (FACS) analysis of whole blood of patients with severe sepsis and septic shock (patients enrolled in paper I). This study of intracellular resistin revealed that the majority of resistin-positive cells in circulation were also neutrophils, indicating that this is likely the source for the pronounced systemic hyperresistinemia in severe sepsis and septic shock patients. Analyses of an extended severe sepsis/septic shock cohort (n=39) revealed a significant correlation between serum resistin levels and neutrophil counts at the inclusion day ($r=0.53$, $p=0.0005$), further strengthening our results. At the same time, we also performed a Triton X-100 lysis of primary neutrophils of healthy donors. There was a marked interindividual variation in the total resistin content ranging from 4 to 10 ng/ 10^6 neutrophils. It is possible that such differences in resistin content could represent a predisposing factor for developing severe infections. The concentrations seen in the circulation (up to 300 ng/ml) of septic shock patients can obviously be readily sustained by the mobilization and degranulation of neutrophils, especially in light of the pronounced neutrophilia, which is common in these patients.

To explore the subcellular location of resistin, cell membranes of neutrophils from healthy donors were disrupted and the resulting cellular content was subjected to density centrifugation on a Percoll gradient. Fractions were collected and investigated by Western blotting or dot blot analysis using antibodies against MPO (a marker protein for azurophilic granules), lactoferrin (a marker for specific granules), and CD35 (a marker for secretory

vesicles). Resistin levels in each fraction were analyzed and revealed resistin in both MPO- and lactoferrin-containing fractions, however at a lower concentration in the latter. In the subsequent study by Bostrom et al. they also reported on resistin in both azurophilic- and specific granules [290]. We explored this finding further, by staining primary neutrophils for resistin and granule marker proteins and evaluation by confocal microscopy. 100% of the resistin-positive granules were double positive for MPO, whereas no colocalization with lactoferrin was seen.

Thus, the results revealed neutrophils as a dominant source of resistin in severe infections and linked resistin to the azurophilic granules within the neutrophils. Azurophilic granules are, of the different granule types, the latest to be mobilized, which could explain the accumulation and persistence of resistin at the infectious site.

4.3.3 Triggers of resistin-release

We have identified neutrophils as a novel source of resistin and to address the trend of differences in resistin responses pending on causative microorganisms seen in the previous study of our group [231], we next performed *in vitro* assays. A given candidate among others to evaluate was the M1-protein. In these assays, we stimulated primary neutrophils of healthy volunteers with either equivalent concentrations of clinical isolates of *E. coli* and GAS, or bacterial factors such as M1 protein or LPS. The highest resistin levels were consistently seen when GAS stimuli (fixed bacteria or M1 protein) were used, whereas LPS or fixed *E. coli* proved to be fairly poor inducers of resistin (figure 15). We now demonstrate that the streptococcal M1 protein is a prominent trigger of resistin release from neutrophils. In addition, we show that the response is dependent on the presence of fibrinogen-containing plasma, implicating the above described mechanism in these responses. However, the question of whether Gram-positive toxic shock is associated with a more pronounced resistin response than Gram-negative septic shock remains to be answered, preferably by analyses of larger patient cohorts enrolled in the same study.

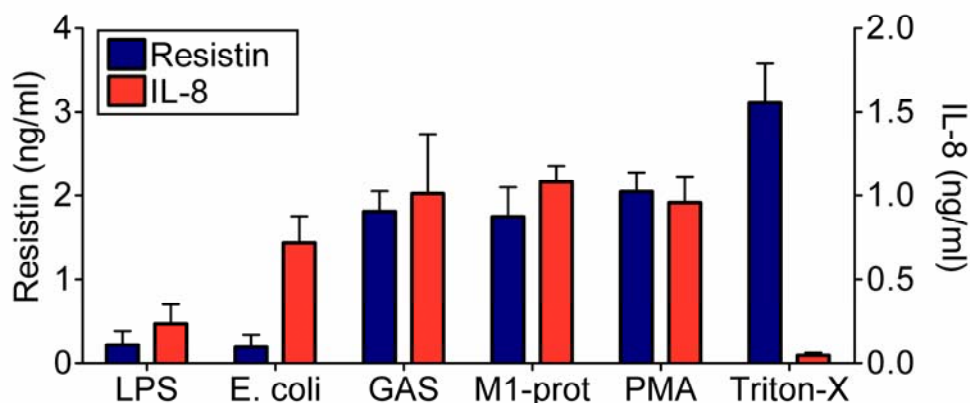


Figure 15. Bacterial triggers of resistin release. Neutrophils were stimulated with fixed bacteria or bacterial factors and resistin and IL-8 were measured in cell supernatant. Fixed GAS and streptococcal M1 protein elicited a higher resistin release than *E. coli* and LPS. Resistin is pre-stored in neutrophils, revealed by Triton-X lysis of the cells (a release of >60% of total resistin content), as compared to IL-8 which is up-regulated during severe infection.

The physiological function and regulation of resistin in human is yet to be further defined. Resistin modulation has been done in only a few studies. *In vitro*, resistin expression is shown to be regulated by peroxisome proliferator-activated receptor γ (PPAR γ) activators and can be blocked by the antidiabetic treatment rosiglitazone, a PPAR γ agonist [224]. In patients with diabetes typ II, the resistin plasma concentrations [291] and gene expression in adipocytes is suppressed by rosiglitazone [292, 293]. Resistin levels were also seen to be suppressed in patients with inflammatory bowel disease treated with infliximab, an anti-human TNF- α monoclonal mouse antibody [294].

There are many plausible mechanisms by which resistin may contribute to sepsis. Future studies will be required to study intracellular mechanisms. However, there are major differences in both mouse and human resistin homology as well as a disparity in the cellular source of resistin making it difficult to obtain clinical relevance of animal studies. Identifying neutrophils as a new cellular source of resistin is nevertheless important and the findings led us to further explore and define the role of resistin in neutrophil activation in normal immune responses and in sepsis. The question whether it is the neutrophil modulation rather than mediator modulation that would be the new target in sepsis remains to be answered.

4.4 BACTERIAL INDUCED NEUTROPHIL ACTIVATION (PAPER IV)

In **paper III**, we identified neutrophils as a novel and dominant source of resistin. The recruitment and activation of neutrophils are critical for the immune defense, since they play an important role in bacterial killing through mechanisms such as phagocytosis, formation of NETs and production of antimicrobial effector molecules. On the other hand, the release of granule proteins stored within the neutrophils can cause cell and tissue damage to the host, as well as dysregulated inflammatory response [295]. The fact that both HBP and resistin are neutrophil-derived proteins elevated in sepsis patients raised the question of their potential correlation and interaction. Since a strikingly difference in resistin release from neutrophils in response to microbial stimuli was observed in paper III, in **paper IV**, we further extend on the studies on whether neutrophil responses differ depending on stimuli and how this relates to sepsis of different aetiology. Our results show that there are marked differences in neutrophil release of the sepsis-associated HBP and resistin upon exposure to various bacterial strains or bacterial factors.

4.4.1 Neutrophil activation - triggers of HBP and resistin-release

With an extended focus on release of both HBP and resistin, we compared the ability of different bacterial stimuli to trigger neutrophil activation and degranulation by exposing primary neutrophils isolated from healthy blood donors to clinical septic shock isolates (originating from **paper I**), namely *E. coli*, *S. aureus* and GAS. Filtered supernatants and fixed bacteria prepared from overnight cultures were used to stimulate neutrophils for 2 hours, after which HBP and resistin were measured in cell culture supernatants. Stimulation with bacterial supernatants failed to induce either HBP or resistin release. In contrast, high levels of HBP and resistin were detected after stimulation with fixed GAS strains (n=4), whereas significantly lower levels were triggered by fixed *S. aureus* (n=2) or *E. coli* strains (n=2) (p<0.001) (Figure 16A and B).

We also performed extended *in vitro* experiments with stimulation of neutrophils with bacterial components including the streptococcal M1-protein and LPS. PMA and fMLP, two general

triggers of neutrophil activation, were used as positive controls. The streptococcal M1-protein resulted in release of significantly higher amounts of both HBP and resistin, as compared to LPS that failed to induce release of either mediator ($p < 0.016$). Previous reports have showed that, although LPS has a priming effect, it is a weak agonist of neutrophil azurophilic granule exocytosis [296], supporting our finding of a poor effect of the TLR-agonist LPS. Thus, these findings were in agreement with the bacterial stimulation experiments. In addition, kinetic experiments revealed a similar profile for HBP and resistin release in response to streptococcal M1 protein, where both factors starting to appear after 30-45 minutes of stimulation.

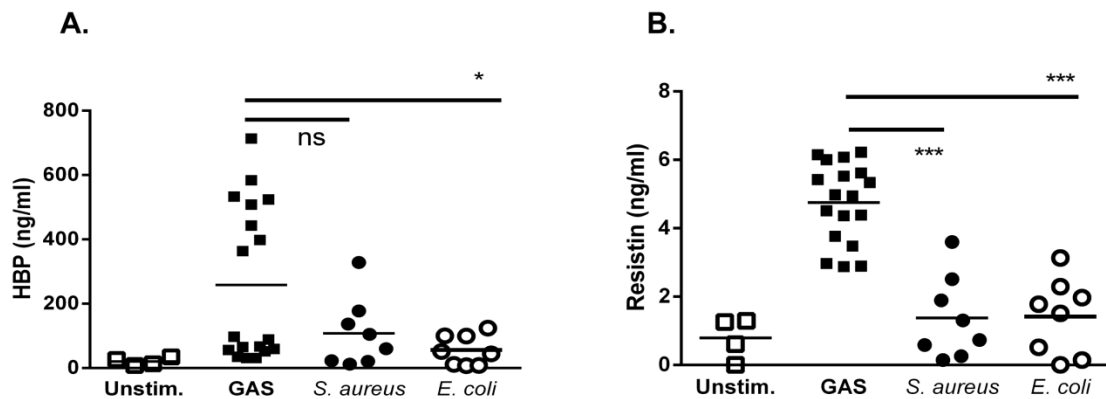


Figure 16. Release of HBP and resistin differs depending on bacterial stimuli, HBP in A and resistin in B.

The neutrophil-stimulatory effect seen in GAS and M1-protein was further explored in other streptococcal species collected from septic shock patients, namely GBS, GCS, GGS, and *S. viridans* strains. Five different GAS strains including strains of serotypes T1, T4 and T28, as well as one strain with unknown type were tested and found to elicit equally strong responses (figure 17). All these strains were found to induce a strong resistin response in the two donors tested, whereas the HBP response was only seen in donor 2.

In conclusion, different streptococcal strains trigger both HBP and resistin. The underlying mechanism remains however to be elucidated. Streptococcal strains commonly express fibrinogen-binding proteins, which in GAS, GBS, GCS and GGS predominantly consists of members of the M/M-like protein family [297]. Our strains included T1 (coupled to *emm1*/M1 serotype), T4 and T28 types, the latter two types both expressing the M-related protein MRP4, similarly to M1-protein, harbouring two fibrinogen binding sites [298]. This dual binding has been proposed to strengthen the efficiency of complex formation and more potent neutrophil activation [106]. Similarly, in *S. viridans*, a phage lysin with fibrinogen-binding capacity has been identified [299]. Fibrinogen-binding proteins of GBS, GCS, GGS and *S. viridans* are plausible candidates responsible for the noted neutrophil activation, but this warrants indeed future studies.

In our study, supernatants prepared from the bacterial cultures failed to induce any HBP or resistin release. This finding suggests that surface-associated factors, such as the M protein, rather than secreted factors are involved in triggering neutrophil degranulation. Of note in a previous report, HBP-release was observed following stimulation with GAS overnight culture supernatants, but only by supernatants containing high levels of streptolysin O (SLO) [300]. It might be possible that the lack of a response induced by supernatants in our study is due to low

concentration of SLO in the supernatants used. Humoral responses in patients show that superantigens and streptolysins are produced, but there are no data pertaining to their concentrations and how the expression of virulence factors *in vitro* compares to that in patients is unclear.

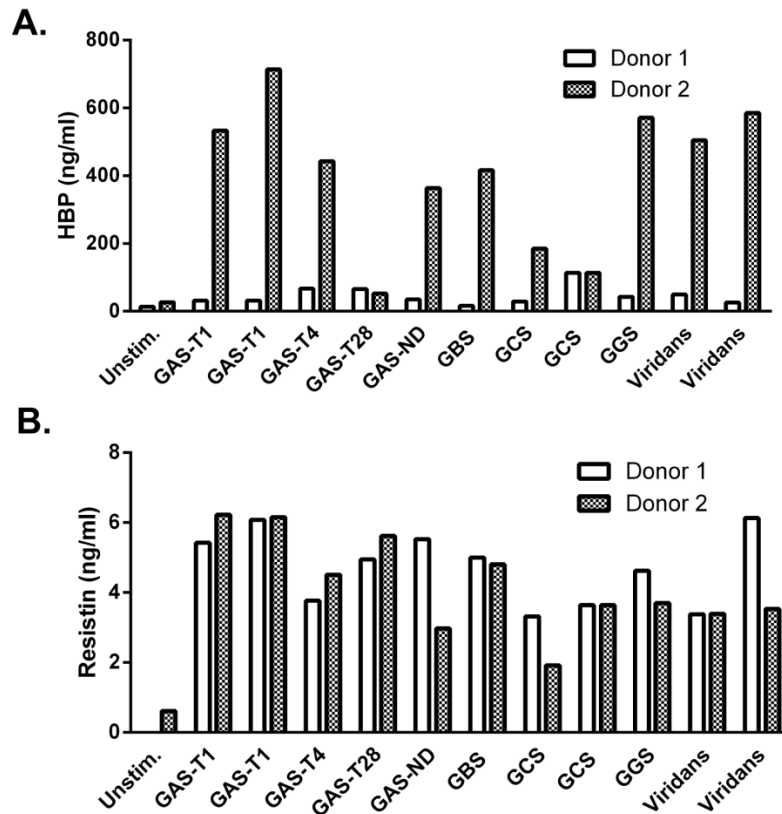


Figure 17. HBP (A) and resistin (B) levels in supernatants from cells stimulated with different streptococcal species as indicated in the figure. ND denotes serotype not determined. Two donors were tested.

Interestingly, with respect to streptococcal-triggered HBP-release, a marked inter-individual variation was observed between cells of different donors. The donors divided into groups of either low or high-responders (figure 16-17 A). In contrast, all donors responded with a high resistin release upon stimulation with fixed GAS strains (figure 16-17 B). Thus, the data indicate that there are differences in mechanisms of activation and/or exocytosis of specific neutrophil-factors. Inter-individual variation in HBP-responses between donors with some being low responders and others high responders has previously been reported by Kahn et al, who also linked the variations in HBP-release upon GAS stimulation to presence or absence of anti-M protein antibodies [301], in which only individuals with high anti-M1 IgG titers were HBP responders. It was proposed that the high response was due to dual engagement of Fc-receptor and β 2-integrin [301]. It seems likely that the donor variation noted in this study might be due to variations in antibodies to streptococcal strains in general.

4.4.2 Subcellular localization of HBP and resistin and potential correlation

In **paper III**, resistin was reported in a heterogenous subset of azurophilic granules [302]. HBP is stored both within the secretory vesicles, which are the most readily mobilized

granules, and the azurophilic granules [99]. In **paper IV**, we analysed HBP and resistin granule localization, since both factors were found to be released by the same stimuli and with similar kinetics, but yet showed a different profile in donor response. Triple intracellular immunostainings of both resting and stimulated cells followed by confocal microscopy were conducted, and analyses of neutrophils for intracellular localization of HBP, resistin and the azurophilic marker MPO were performed. In resting cells, for each factor, numerous positive granules were detected. A large portion of HBP and resistin positive granules also contained MPO, confirming their presence in azurophilic granules, but only few granules were double positive for both HBP and resistin. This finding may in part reflect the previously described heterogeneity in azurophilic granules [303]. Some of the HBP single positive granules may represent secretory vesicles.

When analysing neutrophils activated with various stimuli, different granule staining patterns depending on stimuli were evident. In order to quantify the degree of co-localization, Imaris software analyses were conducted. A 7.5-fold increase in co-localization of HBP with resistin in cells stimulated with streptococcal M1-protein was evident when compared to LPS stimulated cells. In addition, the granules appeared larger in size, which indicates a mobilization of HBP- and resistin-positive granules in response to streptococcal M1-protein. Taken together with the similar kinetic response profile, the data suggest a synchronized release of these two factors in response to GAS. The data is in agreement with the noted differences in *in vitro* responses elicited by various bacterial stimuli, but the molecular mechanisms remain to be elucidated.

4.4.3 Correlation between HBP and resistin in patients

To validate the clinical relevance of these *in vitro* findings, suggesting that the degree of HBP and resistin release from neutrophils is highly dependent on bacterial stimuli, we next evaluated systemic HBP and resistin in severe sepsis/septic shock patients of defined microbial aetiology (described in materials and methods, where patients originated from **paper I**). Septic patients, including both the Gram-positive and the Gram-negative bacterial infections, had significantly higher levels of both factors in acute phase plasma samples when compared to non-infected critically ill patients ($p < 0.001$). No significant differences in HBP or resistin levels between patients infected with Gram-positive ($n=20$) or Gram-negative ($n=28$) bacteria were evident. In this patient cohort from **paper I**, the Gram-positive infections were predominantly caused by *Enterococcus* or *S. aureus*, but no streptococcal strains [253]. We therefore included a separate patient cohort consisting of GAS-infected STSS patients (see materials and methods) and analyses revealed high levels of both HBP and resistin. In all three cohorts we registered significant correlations between HBP and resistin levels. Consistent with our *in vitro* findings, this correlation was stronger in infections caused by Gram-positive ($r=0.65$, $p=0.003$) as compared to Gram-negative bacteria ($r=0.49$, $p < 0.001$) and was particularly striking in the GAS infected cohort ($r=0.8$, $p=0.016$).

We next analyzed the correlation the local site of infection. As previously reported, HBP and resistin are readily detectable in snap-frozen tissue biopsies collected from patients with GAS NF or severe cellulitis compared to only a few cells positive for resistin in skin biopsies from healthy controls [102, 302]. Correlation test revealed an impressive correlation between HBP and resistin at the infected tissue site ($r=0.91$, $p=0.0013$), in agreement with plasma samples.

In line with the previous described *in vitro* findings, the above analyses showed impressively strong correlations between HBP and resistin both locally and systemically ($r=0.9$ and $r=0.8$, respectively). Our findings both *in vitro* and *in vivo* suggest that streptococcal strains are highly

potent triggers of both HBP and resistin. The involvement of streptococcal M-protein, among other factors, mediating β 2-integrin activation in neutrophil triggering and degranulation [304] is most likely a major mechanism resulting in the granule mobilization and exocytosis, but this needs to be further elaborated.

4.4.4 Synergistic effect of HBP and resistin on inflammatory response

The physiological function of the observed co-presence of these two effector molecules is not clear, although both HBP and resistin are two factors associated with severity of sepsis, and both have been reported to exert pro-inflammatory activities [221, 236, 246, 305, 306]. We further studied whether there was a potential interaction between these factors. PBMCs from healthy donors were stimulated with either HBP or resistin alone, or the combination of the two factors, where after IL-8 (a sepsis associated pro-inflammatory cytokine) was measured in culture supernatants. Stimulation of PBMCs with HBP and resistin in combination, when compared to either factor alone, resulted in a significantly higher inflammatory response, as determined by IL-8 release. In addition, IL-8 responses in patients positively correlate with HBP and resistin in STSS patients, not evident in the large sepsis cohort.

Paper IV focuses on the relatively unexplored immunostimulatory effects of neutrophil-pathogen interactions that may contribute to tissue and organ damage. The data support the concept that neutrophil activation varies markedly upon exposure to different bacterial stimuli, and in the case of streptococcal infections this is likely a major contributor to the pathogenesis. Hence, intervention with bacterial-triggered activation may be a novel target for treating these infections. This has important implications for therapeutic strategies, especially considering that several trials have attempted to enhance neutrophil recruitment and function in sepsis. Our data underlines again the importance of personalized medicine and the need for targeted patient groups.

5 CONCLUDING REMARKS AND FUTURE ASPECTS

This two-armed thesis underscores the complexity of both the clinical and pathophysiological aspects of sepsis.

- The clinical study (**paper I**) demonstrated a low mortality in severe sepsis/septic shock, in aspects of both short- and long-term mortality, as compared to reports from outside Scandinavia. Early adequate antibiotic treatment and the low incidence of resistant isolates may partly explain these findings. Our patients matched previous studies in severity of disease, age and underlying co-morbidities. A troubling finding was the trend of women having a delay in administration of antibiotics as compared to men. This finding warrants further research in larger studies and, if validated, should lead to implementations of altered routines in patients care.
- In **paper II**, we evaluated clinical efficacy of IVIG in a comparative observational study of 67 prospectively identified STSS patients, in which patients treated with IVIG had a significant higher survival rate compared to the non-treated patients (87% vs 50%, $p=0.0034$). Multivariate analysis revealed that both IVIG and clindamycin therapy contributed to a significantly improved survival in STSS.
- In **paper III**, high concentrations of resistin were demonstrated both systemically and locally. We identified the neutrophils as a novel dominant source of resistin and the resistin-release was triggered by the streptococcal cell wall components and by the M1 protein but not by streptococcal superantigens or LPS. This report emphasizes the importance of neutrophils as dominant sources of resistin during severe bacterial infections and places resistin among the potentially important neutrophil granule proteins that may contribute to the pathogenesis of inflammatory diseases.
- In **paper IV**, we explored further whether neutrophil responses, in particular the release of the sepsis-associated factors HBP and resistin, differ depending on stimuli and how this relates to sepsis of different aetiology. We revealed a striking variation in neutrophil release of sepsis-associated HBP and resistin upon exposure to different clinical sepsis isolates or bacterial factors, with streptococcal strains and M1-protein being potent triggers of both HBP and resistin release, as compared to *S. aureus*, *E. coli* and LPS. Plasma HBP and resistin levels correlated significantly in septic patients, with the strongest association seen in GAS-infected cases. In addition, combined HBP and resistin stimulation of PBMCs elicited a higher IL-8 response as compared to each protein alone. This study reveals pronounced differences in neutrophil responses to various bacterial stimuli, with streptococcal strains being particularly potent inducers of the sepsis-associated factors HBP and resistin. However, the underlying physiological mechanism is not clear, and remains to be elaborated. Our data support the concept that neutrophil activation is likely a major contributor to the pathology of GAS infections. This may have important implications for therapeutic strategies, and which patient groups should be targeted for neutrophil modulation.

6 SAMMANFATTNING PÅ SVENSKA

Sepsis, eller blodförgiftning, är ett allvarligt och potentiellt livshotande sjukdomstillstånd. Svår sepsis och septisk chock är de allvarligaste formerna av sepsis, förenade med hög dödlighet, med varierande höga mortalitetssiffror runt 25-50%. De flesta som inte arbetar inom sjukvård är fortfarande ganska okunniga om dessa sjukdomar. I en nyligen publicerad studie där man intervjuat 6000 personer hade 88 % aldrig hört talas om sepsis, och bland de 12 % som hade hört ordet förut visste mer än hälften inte om att det var en så allvarlig sjukdom som det faktiskt är [307]. Denna okunnighet står i kontrast till det faktum att sepsis är den andra ledande orsaken till död på våra intensivvårdsavdelningar [308], och den tionde vanligaste dödsorsaken totalt i väst (USA). Detta ger förstås upphov till, inte bara personligt lidande hos patienter och anhöriga, utan också till stora kostnader för samhället, både direkta och indirekta i efterförloppet av sjukdomen. Svår sepsis och septisk chock anses vara orsakade av ett överdrivet immunförsvar hos patienten, med en frisättning av en rad olika ämnen som ger upphov till lågt blodtryck, läckage av vätska från kärlen ut till vävnaden, försämrat syrgasutbyte och slutligen, i värsta fall, organsvikt och död.

Denna avhandling är tvådelad i så måtto att vi har studerat både kliniska och patofysiologiska händelser vid sepsis. I det första delarbetet ville vi kartlägga patienter med svår sepsis och septisk chock som vårdas på intensivvårdsavdelningen på Karolinska Universitetssjukhuset, Huddinge. Vi följde 101 patienter varav majoriteten hade septisk chock. Vi kunde se att våra patienter inte skiljde sig nämnvärt från andra stora studier med hänseende till ålder, svårighetsgrad av sjukdom och underliggande sjukdomar. Vad som däremot var utmärkande var en förhållandevis låg dödlighet, både när det gäller 28 dagars-, sjukhus- och 1-års mortalitet (19 %, 29 % och 34 %). En bidragande orsak till detta är troligen att de flesta patienter fått bred och rätt antibiotikabehandling initialt och att vi inte hade någon större andel multiresistenta bakterier. När vi tittade närmare på de 43 patienterna som kom direkt från akuten till IVA kunde vi se att de flesta fick antibiotika omgående, men att det fanns en könsskillnad i tid till att träffa en läkare på akuten och tid till antibiotikabehandling, till kvinnornas nackdel. Orsaken till detta är inte klarlagd och det krävs större studier för att säkerställa denna observation.

I det andra delarbetet valde vi att titta närmare på en specifik undergrupp av patienter med septisk chock, nämligen de som drabbas av Streptococcal Toxic Shock Syndrome (STSS). Detta är ett ovanligt tillstånd förenat med mycket hög dödlighet, och den orsakande grupp A streptokocken (GAS) benämns ofta i media som "köttätande" eller "mördar-" bakterie. Vi vet att tidig handläggning och snabbt insättande av antibiotika kan rädda liv i kombination med tidig kirurgisk åtgärd i de fall som även lider av det fruktande tillståndet nekrotiserande fasciit (NF), en mycket svår djup hudinfektion. Trots detta är dödligheten mycket hög och man har försökt hitta tilläggsbehandling som kan göra att utgången förbättras. *In vitro* studier och ett fåtal observationella studier har påvisat en ökad överlevnad vid tilläggsbehandling av immunoglobulinterapi (IVIG) men med låg bevisnivå. I en stor svensk övervakningsstudie av invasiva GAS infektioner kunde vi återfinna 67 patienter som led av sjukdomen STSS. Vi delade in patienterna i de som hade respektive inte hade fått behandling med IVIG, och vi kunde se att det var en ökad överlevnad i den gruppen som hade fått IVIG. Vi analyserade också specifika faktorer som kan vara avgörande för överlevnad och kunde i detta material se att både användandet av klindamycin som tilläggsbehandling till vanlig antibiotika och IVIG var signifikanta och viktiga för en bättre prognos. Studien styrker användandet av IVIG som tilläggsbehandling vid det specifika tillståndet STSS.

Kliniska prövningar av behandlingar som minskar enskilda frisatta ämnen vid sepsis har ännu inte lyckats minska dödligheten i dessa svåra sjukdomstillstånd. En förklaring kan vara att frisättningen av dessa ämnen redan är förbi när patienten väl kommer till sjukhus. I jakten på att finna nya markörer och mediatorer vid sepsis och i förlängningen eventuellt kunna hitta målproteiner för tänkbar behandling har vår forskargrupp tidigare fokuserat mycket arbete kring ett protein kallat resistin som har en förlängd utsöndringsprofil jämfört med andra faktorer, i ett skede där eventuell behandling fortfarande kan vara aktuell och genomförbar. I delarbete tre studerade vi resistin vid septisk chock, och STSS och NF i synnerhet. Vi noterade förhöjda värden vid svår sjukdom samt att värdet låg kvar högt länge. Vi analyserade också vävnadsbiopsier från patienter med STSS och kunde se en hög frisättning av resistin på platsen för infektion, samt att neutrofilerna var den största källan till resistinfrisättning både i blodet och i den infekterade vävnaden. Vi genomförde därefter *in vitro* analyser av neutrofiler och vi kunde se en ökad frisättning av resistin när cellerna stimulerades med fixerade bakterier och GAS bakteriens M1-protein till skillnad från gramnegativa LPS, *E. coli* eller streptokockens superantigen. Studien gav oss svar på vilken cell som frisätter resistin i vävnad och cirkulation, i vilken del av den vita blodkroppen som resistin finns och att det föreligger en skillnad i frisättning av resistin beroende på vilket stimuli som finns.

I det sista delarbetet gick vi vidare med vetskapen om att både HBP och resistin frisätts av neutrofiler och ses i förhöjd nivå vid sepsis. Frågan som specifikt ställdes var om de hade någon korrelation eller interaktion med varandra samt om neutrofilsvaret var olika beroende på bakteriellt stimuli och således sepsis av olika etiologiskt ursprung. För detta ändamål stimulerades neutrofiler med olika bakteriella komponenter som grampositiva bakteriens M1 protein, den gramnegativa bakteriens LPS, samt olika kliniska isolat från studie 1. Vi visade att olika streptokockbakterier frisätter en signifikant högre nivå av både HBP och resistin jämfört med *S.aureus* och *E.coli* bakterier. HBP och resistin var klart förhöjda hos patienter med svår sepsis och septisk chock jämfört med en grupp patienter som inte hade infektion men som var svårt sjuka av andra anledningar. Faktorerna korrelerade signifikant hos dessa patienter och den starkaste associationen återfanns hos patienter med GAS infektion. När vi sedan stimulerade blodets celler med kombinationen HBP och resistin sågs ett klart högre inflammatoriskt svar i form av IL-8 jämfört med varje protein för sig. Studien visar att det är stora skillnader i neutrofilaktivering och svar beroende på olika bakteriella stimuli. Vidare visades att olika streptokockbakterier är speciellt potenta frisättare av både HBP och resistin. Vilken specifik del på streptokocken som ansvarar för detta återstår att ta reda på.

Denna avhandling ger nya insikter i sepsisfältet genom att dels sammanställa en viktig deskriptiv bild av dödligheten bland sepsispatienterna på våra intensivvårdsavdelningar, och dels utröna effekten av IVIG som tilläggsbehandling vid STSS. Den ger oss också nya kunskaper om patofysiologin vid sepsis och våra observationer kan förhoppningsvis utgöra en grund för vidare forskning inom sepsisfältet i jakten på nya behandlingar.

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