Biomarkers of Necrotising Soft Tissue Infections

Aspects of the Innate Immune Response

Marco Bo Hansen

This review has been accepted as a thesis together with three original papers by the University of Copenhagen on 17th October 2016 and defended on 4th November 2016.

Tutors: Ole Hyldegaard, Peter Garred, Ulf Simonsen and Lars Simon Rasmussen.

Official opponents: Erik Waage Nielsen (NO), Christian Backer Mogensen (DK) and Kirsten Møller (DK, Chairperson).

Correspondence: Department of Anaesthesia, Centre of Head and Orthopaedics, Rigshospitalet, University of Copenhagen, Denmark.

E-mail: marcobhansen@gmail.com

Dan Med J 2017;64(7):B5389

The thesis was based on the following three original papers:

1. **Hansen MB**, Rasmussen LS, Garred P, Bidstrup D, Madsen MB, Hyldegaard O. Pentraxin-3 as a marker of disease severity and risk of death in patients with necrotizing soft tissue infections: a nationwide, prospective, observational study. Crit Care. 2016;20:40. (**Paper 1**)

2. **Hansen MB**, Rasmussen LS, Pilely K, Hellemann D, Hein E, Madsen MB, Hyldegaard O, Garred P. The lectin complement pathway in patients with necrotising soft tissue infection. J innate Immun. 2016;8(5):507-16. Accepted for publication at time of defence (**Paper 2**)

3. **Hansen MB**, Rasmussen LS, Svensson M, Chakrakodi B, Bruun T, Madsen MB, Perner A, Garred P, INFECT Study Group, Hyldegaard O, Norrby-Teglund A. The association between cytokine response and disease severity in patients with necrotising soft tissue infection: a multicentre, prospective, observational study. Sci Rep. 2017;7:42179. Submitted at time of defence (**Paper 3**)

LIST OF ABBREVIATIONS

AUC	Area under the curve
CI	Confidence interval
CRP	C-reactive protein
DAMP	Damage-associated molecular patterns
ELISA	Enzyme-linked immunosorbent assay
HR	Hazard ratio
ICU	Intensive Care Unit
IL	Interleukin
IQR	Interquartile range
LRINEC	Laboratory Risk Indicator for Necrotizing Fasciitis
MASP	Mannose-associated serine protease
MBL	Mannose-binding lectin
MCP	Monocyte chemoattractant protein
NSTI	Necrotising soft tissue infection
OR	Odds ratio
PAMP	Pathogen-associated molecular pattern
PCT	Procalcitonin
PRM	Pattern recognition molecules
PRR	Pattern recognition receptors
PTX3	Pentraxin-3
ROC	Receiver operating characteristic
RRT	Renal replacement therapy
SAPS	Simplified Acute Physiology Score
SOFA	Sepsis-related Organ Failure Score
TNF	Tumor necrosis factor

1. INTRODUCTION

1.1 Necrotising soft tissue infection

Necrotising soft tissue infection (NSTI) was first described by Hippocrates in the 5th century BC as a complication of erysipelas: "*many were attacked by the erysipelas all over the body when the exciting cause was a trivial accident or a very small wound... Many even while undergoing treatment suffered from severe inflammations, and the erysipelas would quickly spread widely in all directions. Flesh, sinews and bones fell away in large quantities…There were many deaths.*"[1].

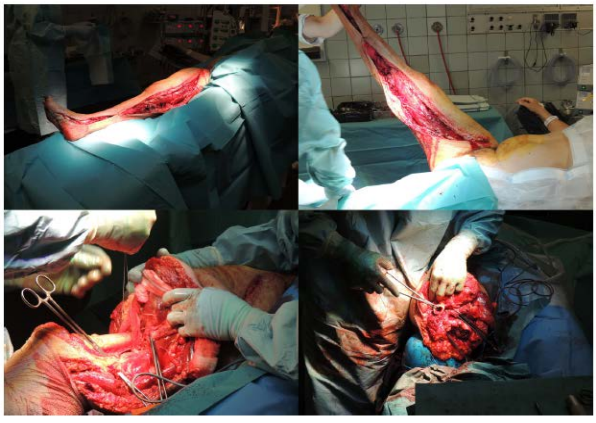

Figure 1. A patient with NSTI located to the leg undergoing above-knee amputation.

Definition

NSTI is a bacterial infection causing necrotic lesions in any layer within the soft tissue compartments[2,3]. NSTI represents the more severe form of soft tissue infections and encompasses necrotising fasciitis, Fournier gangrene, necrotising myositis, and other necrotizing infections[3].

NSTI has been described according to its anatomical location and depth of infection, but these stratifications have limited implication for the treatment. The microbiological aetiology, however, is clinically relevant for the classification of NSTI[4,5]. Type 1 is a polymicrobial infection caused by various species of gram-positive cocci, gram-negative rods, and anaerobes including clostridium species. Type 1 NSTI is the type most frequently reported in the literature[2,4,6,7]. Type 2 involves monomicrobial infection with group A β-haemolytic *streptococci* or combined with staphylococcal species[8], whereas Type 3 is caused by gram-negative marine organisms, most commonly *Vibrio vulnificus,* after puncture wounds from fish or marine insects[9,10].

Epidemiology

The incidence of NSTI has increased over the past decades[11–13] and is estimated to be 40 cases per 1,000,000 person-years with 4.8 deaths per 1,000,000 person-years in the United States[14,15]. Similar data have been reported from the United Kingdom and New Zealand[2,11]. Limited data exist on the disease burden in Denmark, but an active surveillance of invasive group A streptococcal infections showed an incidence of 2.6 cases per 100,000 person-years[16]; skin and soft tissue infections accounted for 26% and NSTI for 6%. The cause of the increasing incidence is unknown, but higher rates of reporting, increasing bacterial virulence, and increasing antimicrobial resistance (or all three combined) have been suggested[8].

Even though NSTI is uncommon, many physicians will encounter at least one case during their career[7]. Predisposing factors include age, male sex, immunosuppression, diabetes mellitus, peripheral vascular disease, chronic renal failure, underlying malignancies, obesity, and intravenous drug use[2,17,18]. Age is associated with mortality, increasing by 4% every year[19]. Delay from onset of symptoms to surgery by more than 24 hours is also associated with mortality[20–22].

Pathogenesis

NSTI is typically caused by toxin-producing bacteria and the extensive inflammatory response is thought to be a main cause of tissue pathology, systemic toxicity, septic shock, multiple organ failure, and mortality[5,19, 23]. However, the underlying pathogenesis of NSTI is poorly understood. In Type 1 NSTI, it is presumed that the bacterial species work synergistically to enhance growth and spread of infection[24]. In Type 2 NSTI, different virulent factors have been identified, such as M- and M-like proteins, that mediate bacterial adherence to the infected tissue and contribute to antiphagocytic properties, colonization, and immune evasion[25–28]. The endo- and exotoxins stimulate the production of cytokines and the toxin-induced inflammatory response causes capillary leakage, vascular occlusion, diminished blood flow, ischaemia, and widespread tissue necrosis[24,29,30]. Furthermore, some investigators have attributed the rapid spreading of bacteria to the release of enzymes such as collagenase, hyaluronidase, streptokinase, and lipase[21,31–34].

Aetiology and clinical presentation

Aetiology of the infection includes trauma, chronic skin infections, dental infections, postoperative infections, animal and insect bites, herpes zoster, and burns[30,35–37]. However, idiopathic aetiology often accounts for more than one in three cases[38]. The predominant sites of infection are extremities and perineum, but the symptoms are often uncharacteristic with erythema, swelling, and pain as the most frequently observed signs[39].

Diagnostics and treatment

The distinction between non-necrotizing infection and NSTI is of major importance because proper management of NSTI includes early diagnosis with prompt and repeated surgical debridement combined with a broad-spectrum antibiotic regimen, haemodynamic support in the intensive care unit (ICU), and in some countries adjuvant treatment with intravenous immunoglobulin and hyperbaric oxygen therapy[8,10,40]. However, the presentation of the disease is often indistinguishable from non-necrotizing infections, thus potentially delaying diagnosis and treatment[39]. On this background, the Laboratory Risk Indicator for Necrotizing Fasciitis (LRINEC) score was developed as a diagnostic scoring system to detect early cases of NSTI[41]. The score has been associated with amputation and mortality[42,43], but other studies have been unable to provide clear associations between the LRINEC score and clinically important outcomes[44–47]. Other diagnostic tests and symptoms in the early phase have been proposed, such as radiographic visualisation of gas in the tissue and presence of crepitus or bullae, but surgical exploration remains the gold standard for diagnosis[8,30,39].

1.2 Innate immune system

In patients with NSTI, successful development of new treatment strategies depends on knowledge of the complex immunological interplay between pathways occurring in the early phase of infection. However, insight into this field is incomplete. Better understanding of the early responding pathways and mechanisms could potentially help identify novel therapeutic targets that can stop further dysregulation of the immune response, thus improving outcome. Much research has been performed in patients with sepsis where the predominant sites of infection are the lungs, abdomen, and urinary tract[48–52]. Presumably, the immunological response differs in patients with NSTI because of differences in the site of the infectious focus and pathogenesis, including the extensive tissue necrosis.

The innate immune system comprises the first line of defence against invading microorganisms and plays a pivotal role in the period between the initial infection and the establishment of an adaptive immune response[53]. Upon infection, pattern recognition molecules (PRMs) and receptors (PRRs) recognise and bind to structures on the surface of the microorganisms called pathogen-associated molecular patterns (PAMPs) or damaged self called damage-associated molecular patterns (DAMPs), prompting the inflammatory response and systemic release of cytokines, such as interleukins (IL) and tumor necrosis factor (TNF)[54–56].

Complement system

The complement system is a powerful effector arm of the innate immune defence and is a collection of around 40 proteins located in serum and on cell surfaces[57,58]. The complement system is initiated through the classical, lectin, and alternative pathways[59]. The three pathways converge with the formation of C3 convertases, which leads to the generation of effector molecules involved in opsonisation, chemotaxis, cellular lysis, cytokine production, and inflammation[59] (Fig. 2). The complement system contributes to tissue homeostasis and is of special interest in patients with NSTI as necrotic tissues lack the regulatory molecules that in normal tissues prevent the binding of complement[58]. Additionally, accumulating evidence indicates that the complement activation is an important contributor to the tissue necrosis that follows ischaemia[58].

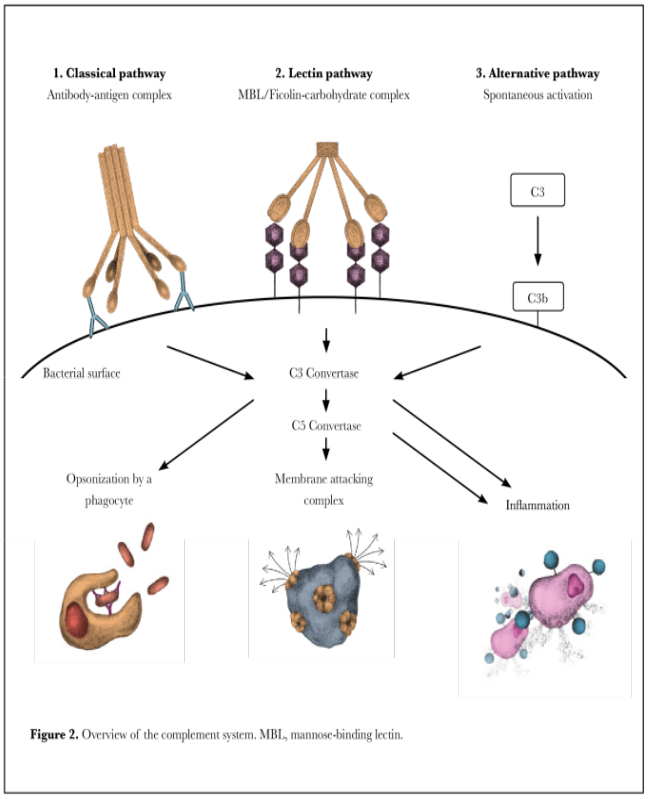

Figure 2. Overview of the complement system. MBL, mannose-binding lectin.

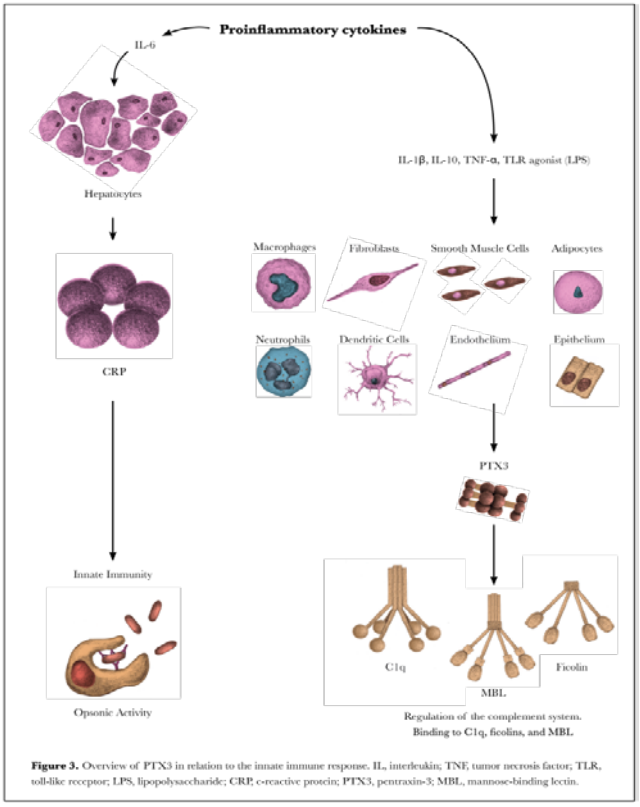

Figure 3. Overview of PTX3 in relation to the innate immune response. IL, interleukin; TNF, tumor necrosis factor; TLR, toll-like receptor; LPS, lipopolysaccharide; CRP, c-reactive protein; PTX3, pentraxin-3; MBL, mannose-binding lectin.

Molecules of the lectin complement pathway and their inflammatory role

The contribution of the lectin complement pathway to defence against bacterial infections has been assessed in several studies, but the exact mechanisms of the effector molecules remain to be elucidated[60]. Mannose-binding lectin (MBL) and Ficolins are a group of PRMs that bind to microbial carbohydrates and activate the complement system through the lectin complement pathway[57]. MBL is produced in the liver[61]; Ficolin-1 in peripheral neutrophils and monocytes and epithelial cells in the lungs[62,63]; Ficolin-2 in the liver[64]; and Ficolin-3 in the bile duct, liver, and lungs[65,66]. MBL deficiency is associated with bacterial infections[67,68], but limited data exist regarding ficolins and bacterial infections[63,69]. It has been shown that ficolins bind to a wide spectrum of microorganisms, suggesting that ficolins are involved in host defence against a wide range of microbial infections[60], but whether this is of clinical relevance in patients with NSTI is unknown.

Pentraxins are multifunctional PRMs that participate in the innate immune defence through clearing of dying host cells and fighting certain microorganisms[70]. Based on the structure, they are divided into short and long pentraxins with C-reactive protein (CRP) classified as a short pentraxin and pentraxin-3 (PTX3) as a long pentraxin[71]. CRP and PTX3 are both acute phase proteins released at the onset of inflammation. CRP is produced in the liver in response to locally induced IL-6, while PTX3 is primarily produced by myeloid cells and expressed in response to inflammatory cytokines, such as IL-1β, IL-10, and TNF-α[72] (Fig. 3). Interestingly, PTX3 has been shown to cross-activate the classical and lectin complement pathways by interacting with C1q, MBL, Ficolin-1, and Ficolin-2[73–75], suggesting the PRMs play an integrated role in fighting invading microorganisms.

Another group of PRMs are the Toll-like receptors that recognize bacterial products[76]. After activation of their microbial ligands, they elicit antimicrobial and inflammatory responses, activating tissue-resident macrophages to produce pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 that generate local and systemic inflammatory responses[76].

1.3 Prognostic biomarkers in patients with NSTI

The prognosis of NSTI depends on accurate diagnosis and immediate initiation of appropriate treatment. Thus identifying patients at highest risk of death may have important therapeutic implications.

Cytokines

Only two small prospective studies have investigated the systemic cytokine response in patients with NSTI[77,78]. Lungstras-Bufler et al. examined serum from 15 patients and found that baseline levels of IL-1β, IL-1Ra, IL-18, interferon-γ, and white blood cell counts were significantly elevated in patients with fatal outcome compared with survivors with NSTI, whereas no differences were observed between patient groups for IL-6 and IL-8[77]. In contrast, Rodriquez et al. examined serum from 18 patients with NSTI and found that they had lower baseline IL-1β levels and higher white blood cell counts compared with patients with cellulitis and/or abscess, but they found no differences in IL-1Ra, IL-6, IL-8, IL-18, and interferon-γ[78].

C-reactive protein and procalcitonin

CRP and procalcitonin (PCT) are used in the ICUs to monitor infectious disease progression in patients with NSTI. However, the current knowledge and scientific rationale are based on only few retrospective studies. Moore et al. investigated 134 patients with NSTI and found that high baseline CRP level was inversely corre-

lated with mortality, but 32% of the values were missing[79]. In contrast, Kincius et al. investigated 41 patients with Fournier gangrene and found significantly higher baseline CRP levels in non-surviving patients[43]. Recently, a retrospective study found that baseline PCT levels correlated with disease severity in patients with NSTI[80], and another study found PCT in combination with the advanced Acute Physiology and Chronic Health Evaluation II score to be a predictor of effective surgical treatment of patients with NSTI[81]. Other studies have identified elevated serum creatinine, sodium, and lactate levels, but not CRP level, as associated with an increased mortality[17,82–86].

2. OBJECTIVES

This thesis seeks to increase the insight on the aspects of the innate immune response in patients with NSTI on admission to hospital, focusing on the inflammatory biomarkers as prognostic markers of disease severity and risk of death. Accordingly, we performed three studies with the following research questions, objectives, and hypotheses:

- *Can PTX3 be used as a prognostic biomarker in patients with NSTI?*
  The objective of this study was to assess plasma PTX3 as a marker of disease severity and mortality in patients with NSTI. The primary analysis focused on the association between PTX3 level upon hospital admission (baseline) and septic shock. We hypothesized that baseline plasma PTX3 level above the median was associated with the presence of septic shock. Secondarily, we focused on the association between baseline PTX3 level and RRT, amputation, 180-day mortality, and long-term mortality up to 2.5 years.

- *Are PRMs of the lectin complement pathway associated with mortality in patients with NSTI?*
  The objective of this study was to investigate the association between plasma PRM levels of the lectin complement pathway and mortality in patients with NSTI. The primary analysis focused on the association between baseline PRMs of the lectin complement pathway and 28-day mortality. We hypothesized that baseline level of PRMs below the median was associated with 28-day mortality. Secondarily, we focused on the association between baseline levels of PRMs and disease severity scores (LRINEC, SAPS II, SOFA) and long-term mortality up to 2.7 years. Lastly, we investigated the degree of complement pathway activation.

- *Can inflammatory cytokines be used as severity markers in patients with NSTI?*
  The objective of this study was to evaluate the association between plasma levels of inflammatory cytokines and disease severity and mortality in patients with NSTI. The primary analysis focused on the association between baseline IL-6 level and the LRINEC score. We hypothesized that baseline IL-6 level above the median was associated with a LRINEC score ≥ 6. Secondarily, we focused on the association between baseline cytokine levels and disease severity scores (SAPS II and SOFA), septic shock, β-haemolytic streptococcal infection, RRT, amputation, and 30-day mortality.

3. METHODS

Detailed information about the study design, materials, and sample processing is available in the original manuscripts. The following gives a brief outline of the studies.

3.1 Overall study design

To test the hypotheses, we conducted three prospective, observational studies. The protocol of the studies has been published[87] and is registered at ClinicalTrials.gov (NCT02180906). The studies are part of the EU project titled INFECT (NCT01790698), which is a consortium of 14 international partners working to advance the understanding of the pathogenesis of NSTI and improve the treatment of these patients (http://www.fp7infect.eu/).

In Studies 1 and 2, we included 135 patients with NSTI at Copenhagen University Hospital, Rigshospitalet, over a 25-month period with a maximum follow-up of 2.5 and 2.7 years, respectively (Table 1). In Study 3, we included 159 patients with NSTI at five Scandinavian hospitals over a 33-month period (Table 1). In Denmark, the treatment of NSTI has been centralised at a national level. This is not the case in Sweden and Norway where different regions of the countries differ in policies, procedures and routines.

3.2 Data collection and severity scores

Trial investigators or their co-workers extracted the clinical data from the patient medical records and vital status from national registries. All data were entered into a centralised online-database and validated regularly.

The LRINEC score was calculated from 6 variables (glucose, haemoglobin, CRP, white cell count, creatinine, sodium), high scores indicating a high risk of NSTI (range: 0–13)[41]. The Simplified Acute Physiology Score II (SAPS II) was calculated from 17 variables, higher scores indicating more severe disease (range: 0–163)[88]. The Sepsis-related Organ Failure Assessment (SOFA) score was calculated from five sub-scores (0 to 4) according to the respiratory and circulatory system, coagulation, liver, and kidneys, higher scores indicating more severe organ failure (range: 0–24)[89]. The presence of septic shock was defined as the use of vasopressors according to Bone et al.[90].

Table 1. *Method summary and overview of study populations.*

	Study 1	Study 2	Study 3
Study design	Prospective, observational	Prospective, observational	Prospective, observational
Centre of inclusion	Copenhagen University Hospital, Rigshospitalet	Copenhagen University Hospital, Rigshospitalet	• Copenhagen University Hospital, Rigshospitalet, Denmark • Karolinska University Hospital, Stockholm, Sweden • Blekingesjukhuset, Karlskrona, Sweden • Sahlgrenska University Hospital, Gothenburg, Sweden • Haukeland University Hospital, Bergen, Norway
Inclusion criteria	• Diagnosis of NSTI based on surgical findings of necrosis • ≥ 18 years • Admitted to the ICU or undergone surgery for NSTI at Rigshospitalet	• Diagnosis of NSTI based on surgical findings of necrosis • ≥ 18 years • Admitted to the ICU or undergone surgery for NSTI at Rigshospitalet	• Diagnosis of NSTI based on surgical findings of necrosis • ≥ 18 years • Admitted to the ICU or undergone surgery for NSTI at one of the five centres
Exclusion criteria	Diagnosis could not be confirmed during surgery	Diagnosis could not be confirmed during surgery	Diagnosis could not be confirmed during surgery
Inclusion period	Feb 2013–March 2015	Feb 2013–March 2015	Feb 2013–Nov 2015
Patients included	135	135	159
Analysis technique	ELISA	ELISA	Multiplex

NSTI, necrotising soft tissue infection; ICU, intensive care unit; ELISA, enzyme-linked immunosorbent assay.

3.3 Blood sampling

We collected the blood samples from an arterial line into EDTA vacuum tubes upon admission and on Day 3. The patients in Denmark also had blood sampled on Day 1 and Day 2. The blood samples were immediately put on ice until centrifugation. Centrifugation was performed no later than 40 minutes after blood sampling at 3,500 rpm for 10 minutes. The plasma samples were apportioned into 1 mL aliquots and subsequently stored at -80 ˚C until processing.

3.4 Sample processing

PTX3 and PRM measurements (Studies 1 and 2) were performed in May 2015, thus two months after enrolment of the final patient with NSTI (Table 1). Cytokine measurements (Study 3) were performed three months after enrolment of the final patient in February 2016. We used two different methods of analysis in the experimental settings to analyse the biomarkers; sandwich enzyme-linked immunosorbent assay (ELISA) was applied in Studies 1 + 2 and multiplex bead array assay in Study 3. Both methods are widely used and validated in terms of measuring molecules in plasma[91]. ELISA is a highly quantitative and reproducible method, but limited by its ability to measure only one biomarker at a time in a given sample. In contrast, a multiplex assay can measure multiple biomarkers in the same sample at the same time with less sample volume needed. The general principles of a classic sandwich ELISA and multiplex are illustrated in Figure 4 and Figure 5, respectively.

ELISA measurements

Levels of PTX3[92,93], PRMs[63,94–96], and residual complement activation capacity[97,98] were assessed with ELISA developed in the Laboratory of Molecular Medicine, Department of Clinical Immunology, Rigshospitalet. In brief, microtiter plates were coated with catching antibodies for each biomarker of interest followed by incubation of diluted EDTA plasma samples from patients with NSTI. Subsequently, biotinylated detection antibodies against the specific biomarkers were applied to the wells and incubated. We then added an enzyme-conjugated substrate (e.g. streptavidin-horseradish peroxidase conjugate). The plates were developed with a colour reaction through enzyme catalysis using OPD substrate and hydrogen peroxide. The plates were washed between each step to ensure removal of unattached molecules. Measurements of PTX3 and PRM levels were performed with automated analysis in a 384-well format using Biomek FX platform (Beckman Coulter, Fullerton, CA, USA), whereas residual complement activation capacity was measured manually in a standard 96-well plate. Samples were tested in duplicate against a standard pool with known concentration and read on a Multimode Detector system (DTX880) at 490 nm. Based on regression analysis of the standard pool, a best fit equation was generated enabling the conversion of OD values into biomarker levels.

Multiplex measurements

Cytokine levels (IL-1β, IL-6, IL-10, and TNF-α) were measured using multiplex bead kits (Bio-Rad Laboratories, Hercules, CA, USA; Bio-Plex Pro™ assay). In brief, capture antibody-coupled magnetic beads were incubated with diluted patient samples. The plates were subsequently washed and incubated with biotinylated detection antibodies. After washing, the plates were incubated with a reporter streptavidin-phycoerythrin conjugate and analysed using the Bio-Plex® MAGPIX™ Multiplex Reader (Bio-Rad Laboratories, Hercules, CA, USA). The samples were tested in duplicate against a standard pool with known concentration and the mean values were calculated after correction for the diluting steps.

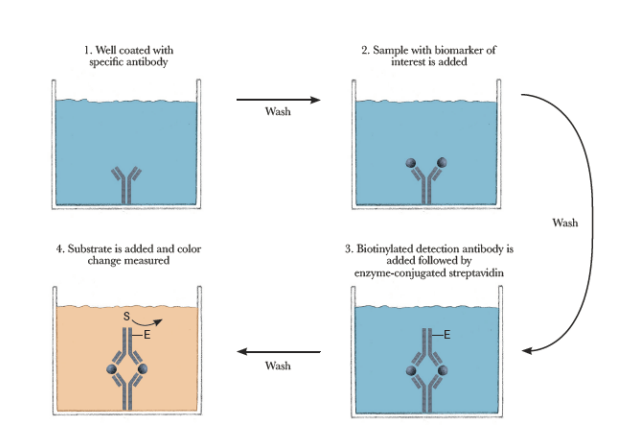

Figure 4. Illustration of the general ELISA principles.

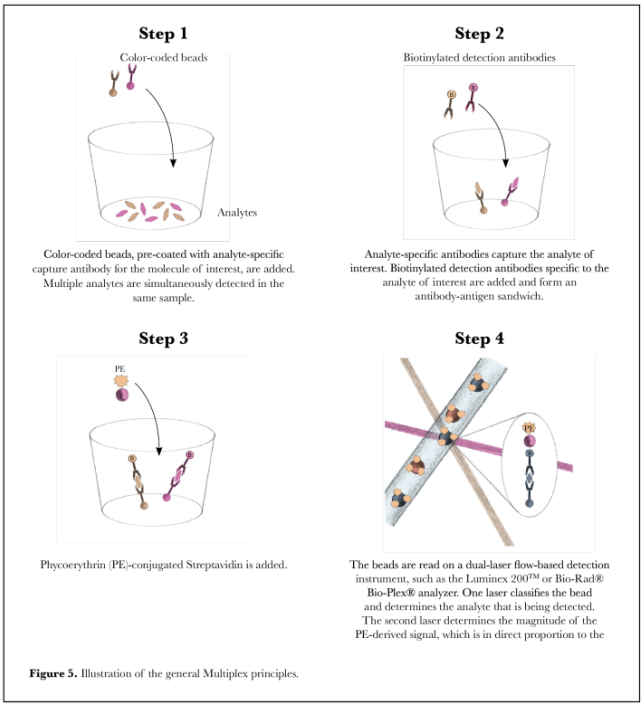

Figure 5. Illustration of the general Multiplex principles.

3.5 Analyses

Study 1

The primary analysis was the comparison of baseline PTX3 level in patients with NSTI with and without septic shock upon admission to Copenhagen University Hospital, Rigshospitalet. The secondary analyses included the association between PTX3, PCT, and CRP levels and disease severity measured by amputation of a body part within the first 7 days of hospital admission, 180-day mortality, and long-term mortality up to 2.5 years.

Study 2

The primary analysis was the comparison of baseline plasma PRM levels of the lectin complement pathway and 28-day mortality in

patients with NSTI. The secondary analyses included the association between baseline PRM levels and disease severity scores (LRINEC, SAPS II, SOFA) and long-term mortality up to 2.7 years. We also analysed the association between the degree of complement pathway activation and 28-day mortality.

Study 3
The primary analysis was the comparison of baseline IL-6 level in patients with NSTI with a LRINEC score < vs. ≥ 6. The secondary analyses included the association between baseline IL-1β, IL-6, IL-10, and TNF-α level and disease severity measured by SAPS II, SOFA score, presence of septic shock (Day 1), β-haemolytic streptococcal infection, use of renal replacement therapy (RRT) in the ICU, amputation of a body part within the first 7 days of hospital admission, and 30-day mortality.

For all three studies, the biomarkers were also compared with non-infected, surgical control patients. For simplicity and due to focusing on the most clinically relevant results, I have chosen not to describe the control cohort and the related analyses and results in the thesis. Please refer to the accompanying manuscripts for further details.

3.6 Statistics
Kolmogorov-Smirnov and Shapiro-Wilk normality tests were performed. Nonparametric continuous data are reported as median (interquartile range, IQR) and comparisons performed using Mann-Whitney *U* test for unpaired analyses and Wilcoxon-rank test for paired analyses. For the primary analyses, we applied the Student *t*-test and used 95% confidence interval (CI) for quantification of differences in biomarker levels between the groups. Categorical data are presented as absolute numbers (%) and compared with χ^2-test or Fisher's exact test. For correlation assessments, we used Spearman's rank correlation, expressed by rho, due to the non-parametric nature of data.

We followed all included individuals until death, emigration, or end of follow-up. A Cox proportional hazards regression model (hazard ratio, HR) or logistic regression analysis (odds ratio, OR) was used to assess the association between baseline level of the biomarkers and all-cause death. The model was adjusted for predefined risk factors: age, sex, chronic disease (diabetes, liver cirrhosis, chronic kidney disease, cardiovascular disease, chronic obstructive pulmonary disease, peripheral vascular disease, immune deficiency, malignancy, rheumatoid disease) and SAPS II. Individuals with missing SAPS II data were excluded from the part of the regression model where adjustment for this covariate was performed. The prognostic value of the biomarkers for long-term mortality up to 2.5 and 2.7 years was investigated using the log-rank test and illustrated by Kaplan-Meier curves. Receiver operating characteristic (ROC) curves were analysed for short-term mortality and used to identify optimal cut-off values (maximum sum of sensitivity and specificity). All analyses were performed using Statistical Package for the Social Sciences 22.0 software (SPSS Inc., Chicago, Ill, USA) and GraphPad Prism 6.0 software (GraphPad Inc., La Jolla, Calif., USA). A two-tailed *p*-value < 0.05 was considered statistically significant.

Sample size
In Study 1, a difference in baseline PTX3 plasma level of 90 ng/mL was considered as clinically relevant as previous studies have found similar differences between patients with sepsis and shock[92,99,100]. Assuming a standard deviation of 100 pg/mL, detection of the desired difference would require inclusion of 52 patients with a statistical power of 90% at the 5% significance level. We needed to include at least 82 patients because we expected the groups to be unequal in size in a ratio of 1:4.

In Study 2, the sample size was determined on the planned recruitment of at least 82 patients with NSTI based on the calculation described above.

In Study 3, a difference in baseline IL-6 plasma level of 1,000 pg/mL was considered as clinically relevant based on previous studies, which found similar differences between patients with sepsis according to shock[101,102]. We found a standard deviation in baseline plasma IL-6 level of 2,000 pg/mL in a pilot study of seven patients with NSTI. Accordingly, we decided to include 159 patients because this would allow us to detect a difference of 1,000 pg/mL with a statistical power of at least 85% at the 5% significance level. We randomly selected 159 patients with NSTI from the entire cohort using a computer-generated list (Figure 11).

3.7 Ethical Considerations
The studies were approved by the ethics committees and data-protection agencies in Denmark (1211709; H-2-2014-071), Sweden (930-12), and Norway (2012/2227). All individuals or their next of kin gave oral and written informed consent.

4. RESULTS
This section presents a summary of results with focus on the primary analyses and clinical outcomes. Tables and figures are modified according to the scope of this section. Detailed results are given in the accompanying manuscripts.

4.1 Study 1: Pentraxin-3
We treated 165 patients with suspected NSTI during the study period. We were not able to obtain blood samples in 3 patients and the diagnosis could not be confirmed in 27 patients. Thus, we analysed PTX3 level in 135 patients (Fig. 6). SAPS II could not be calculated in 5 patients (3.7%) because of missing values.

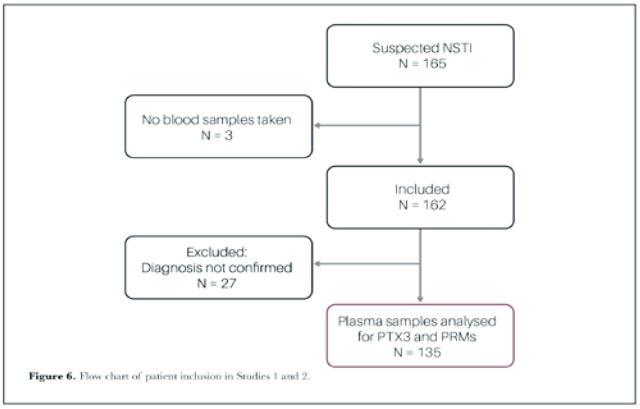

Figure 6. Flow chart of patient inclusion in Studies 1 and 2.

Primary analysis
Patients with septic shock had a significantly higher baseline PTX3 level (Fig. 7). As an elaboration on this (not included in the manuscript), patients with septic shock had a mean baseline PTX3 level of 126.8 ng/mL, whereas those without septic shock had a mean baseline PTX3 level of 51.6 ng/mL (mean difference 75.3 ng/mL, 95% CI [42.7 to 107.8], $p < 0.0001$).

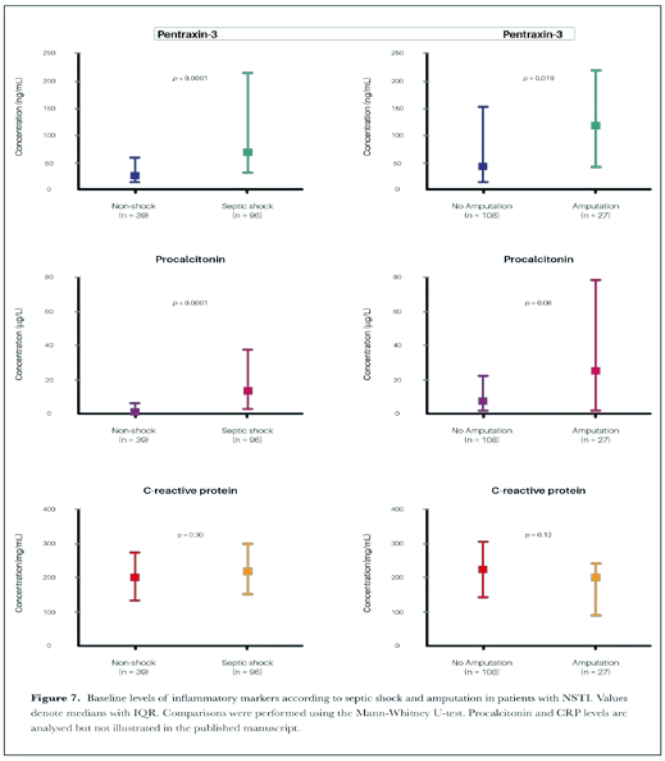

Figure 7. Baseline levels of inflammatory markers according to septic shock and amputation in patients with NSTI. Values denote medians with IQR. Comparisons were performed using the Mann-Whitney U-test. Procalcitonin and CRP levels are analysed but not illustrated in the published manuscript.

Disease severity and mortality

We found a significant difference in baseline PTX-3 and PCT levels according to septic shock, whilst only PTX3 level was significantly higher in patients needing amputation (Fig. 7). Moreover, baseline PTX3 and PCT levels correlated with the disease severity scores (Table 2).

Table 2. *Spearman rank correlation between disease severity scores and baseline biomarker levels in patients with NSTI.*

	PTX3		PCT		CRP	
	Rho	*p*	Rho	*p*	Rho	*p*
LRINEC	0.20	0.029	0.22	0.015	0.24	0.009
SAPS II	0.45	<0.0001	0.47	<0.0001	-0.18	0.04

LRINEC, laboratory risk indicator for necrotising fasciitis; SAPS II, simplified acute physiology score II; SOFA, sepsis-related organ failure assessment; PTX3, pentraxin-3; PCT, procalcitonin; CRP, c-reactive protein. Correlation analyses of PCT and CRP levels are not included in the published manuscript.

During the first 180 days after admission, 36 (27%) patients died. The univariate survival analysis revealed that baseline PTX3, PCT, and CRP levels were significantly associated with 180-day mortality (Table 3). The associations were still significant for PTX3 and PCT when adjusted for age, sex, and chronic disease. However, the associations were not significant when SAPS II was included in the analysis (Table 3). In addition, the diagnostic accuracy for predicting 180-day mortality was low for PTX3 (AUC = 0.66, [95% CI, 0.56-0.76]), PCT (AUC = 0.65, [95% CI, 0.55-0.76]), and CRP (AUC = 0.32, [95% CI, 0.21-0.43]).

Table 3. *Cox regression analysis of mortality up to Day 180 (time of censoring) in patients with NSTI based on high vs. low concentrations of the biomarkers according to median values.*

Please see the original article for results.

4.2 Study 2: Pattern recognition molecules

Patient enrolment followed the flow as described in Figure 6 and constituted the same cohort as in Study 1.

Primary analysis

Those patients who died within the first 28 days after admission had significantly lower baseline Ficolin-2 and Ficolin-3 levels (Fig. 8). We found no difference in MBL and Ficolin-1 levels according to mortality.

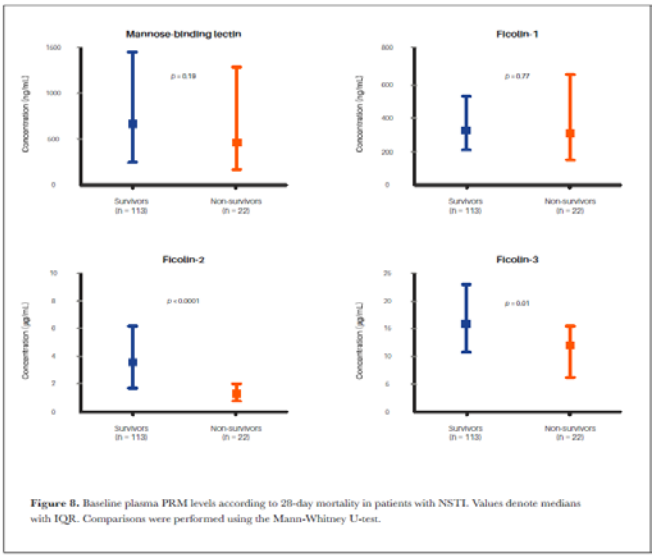

Figure 8. Baseline plasma PRM levels according to 28-day mortality in patients with NSTI. Values denote medians with IQR. Comparisons were performed using the Mann-Whitney U-test.

Disease severity and mortality

Only Ficolin-2 correlated significantly with SAPS II, and MBL with the SOFA score (Table 4). We found no correlation between the plasma levels of Ficolin-1 and Ficolin-3 and the disease severity scores (Table 4).

Table 4. *Spearman rank correlation between disease severity scores and baseline PRM levels in patients with NSTI.*

	MBL		Ficolin-1		Ficolin-2		Ficolin-3	
	Rho	*p*	Rho	*p*	Rho	*p*	Rho	*p*
LRINEC	-0.08	0.40	0.12	0.18	0.09	0.33	0.08	0.38
SAPS II	-0.10	0.25	-0.07	0.45	-0.26	0.002	-0.02	0.85
SOFA	-0.18	0.046	-0.10	0.27	-0.17	0.06	0.02	0.82

LRINEC, laboratory risk indicator for necrotising fasciitis; SAPS II, simplified acute physiology score II; SOFA, sepsis-related organ failure assessment; MBL, mannose-binding lectin.

The univariate survival analysis revealed that baseline Ficolin-2 and Ficolin-3 levels were significantly associated with 28-day mortality (Table 5). The associations remained significant when adjusted for age, sex, and chronic disease, but not when SAPS II was included in the analysis. When the optimal cut-off was used for dichotomisation instead of the median, Ficolin-2 proved to be a significant predictor of 28-day mortality (Table 5). In continuation, the diagnostic accuracy for 28-day mortality was the highest for baseline Ficolin-2 level (AUC = 0.75, [95% CI, 0.63-0.86]) compared with MBL (AUC = 0.59, [95% CI, 0.46-0.72]), Ficolin-1 (AUC = 0.52, [95% CI, 0.38-0.66]), and Ficolin-3 (AUC = 0.67, [95% CI, 0.54-0.80]).

Table 5. *Cox regression analysis of mortality up to Day 28 (time of censoring) in patients with NSTI based on high vs. low concentrations of the biomarkers according to median values.*

Please see the original article for results.

Moreover, a baseline Ficolin-2 level below median was significantly associated with long-term mortality (Fig. 9). Lastly, non-survivors had significantly lower residual activation capacity of the ficolin-mediated lectin pathway upon hospital admission (Fig. 10).

Please see the original article for results.

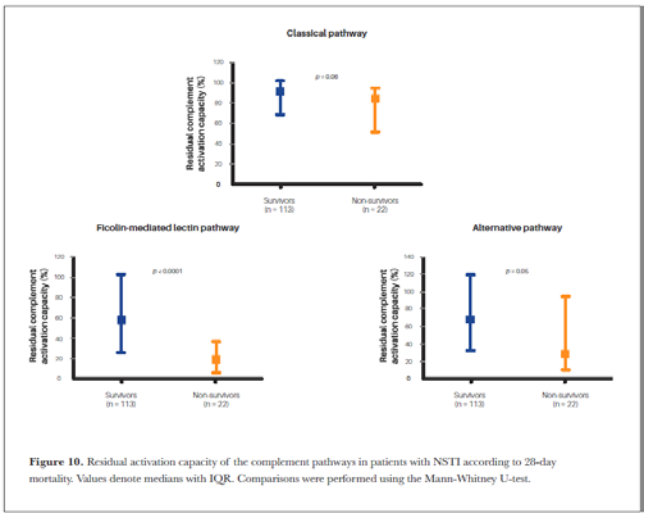

Figure 10. Residual activation capacity of the complement pathways in patients with NSTI according to 28-day mortality. Values denote medians with IQR. Comparisons were performed using the Mann-Whitney U-test.

4.3 Study 3: Inflammatory cytokines

We enrolled 270 patients with NSTI during the study period; 202 patients had data available for the calculation of a LRINEC score. Of these patients, 159 had baseline plasma cytokine level measured (Fig. 11). One foreign patient was lost to follow-up as he left the country on Day 7 and was thus excluded from the adjusted survival analysis. Further, SAPS II could not be calculated in 31 patients (19.5%) because of missing values.

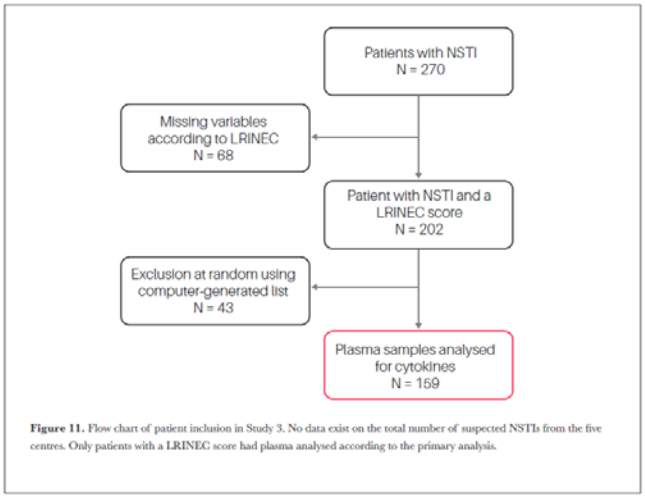

Figure 11. Flow chart of patient inclusion in Study 3. No data exist on the total number of suspected NSTIs from the five centres. Only patients with a LRINEC score had plasma analysed according to the primary analysis.

Primary analysis

One hundred sixteen (73%) patients had a LRINEC score ≥ 6 and a mean baseline IL-6 level of 8,006 pg/mL compared with 10,729 pg/mL in patients with a score < 6 (mean difference 2,723 pg/mL, 95% CI [-6,947 to 12,393], p = 0.58). We did not observe any significant difference between the groups in any of the four baseline cytokine levels (Fig. 12)

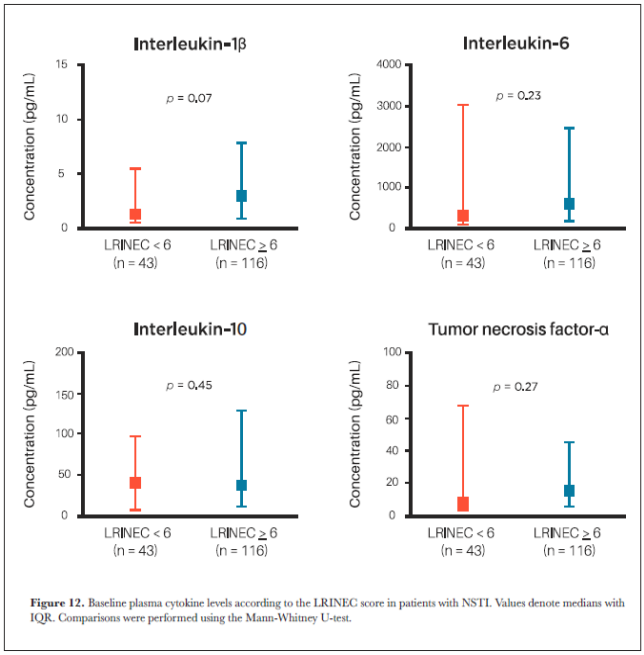

Figure 12. Baseline plasma cytokine levels according to the LRINEC score in patients with NSTI. Values denote medians with IQR. Comparisons were performed using the Mann-Whitney U-test.

Disease severity and mortality

All cytokines showed a significant correlation with the disease severity scores of SAPS II and SOFA with IL-6 demonstrating the highest correlation (Table 6). In contrast, none of the cytokines correlated significantly with the LRINEC score.

Table 6. *Spearman rank correlation between disease severity scores and baseline cytokine levels in patients with NSTI.*

Please see the original article for results.

Patients who received RRT or underwent amputation had higher baseline cytokine levels, except for higher IL-1β levels according to amputation (Table 7). Interestingly, patients with β-haemolytic streptococcal infection had consistently higher baseline IL-6 level compared with other subgroups of infections (Fig. 13).

Table 7. *Differences in median baseline cytokine levels and disease severity in patients with NSTI.*

Please see the original article for results.

Figure 13. *. Baseline levels of plasma cytokines on admission according to microbial aetiology in patients with NSTI. Values denote medians with IQR. Comparisons were performed using the Mann-Whitney U-test.*

Please see the original article for results.

During the first 30 days after admission, 25 (16%) patients died. The univariate logistic regression analysis revealed that high cytokine levels were significantly associated with 30-day mortality (Table 8). The associations remained significant when adjusted for age, sex, and chronic disease. Only IL-1β remained significant when SAPS II was included in the analysis. The diagnostic accuracy for 30-day mortality was almost the same for IL-1β, IL-6, and IL-10 (AUC = 0.70, [95% CI, 0.58-0.82]) and lower for TNF-α (AUC = 0.68, [95% CI, 0.55-0.81]).

Table 8. *Univariate and multivariate logistic regression analyses of mortality up to Day 30 (time of censoring) in patients with NSTI based on high vs. low concentrations of the cytokines according to median values.*

Please see the original article for results.

5. DISCUSSION

5.1 Principal findings

In Study 1, we found that baseline plasma PTX3 level above the median was significantly associated with the presence of septic shock, amputation within the first 7 days of admission, and 180-day mortality. However, PTX3 was not an independent predictor of 180-day mortality.

In Study 2, we found that baseline Ficolin-2 level below the median was significantly associated with 28-day mortality and long-term mortality up to 2.7 years. Ficolin-2 also correlated with SAPS II. Baseline MBL and Ficolin-3 levels were only associated with 28-day mortality. Finally, those patients who died within the first 28 days after admission had significantly lower residual activation capacity of the ficolin-mediated lectin complement pathway.

In Study 3, we found no significant association between the LRINEC score and baseline cytokine levels. IL-6 had the strongest correlation with the disease severity scores (SAPS II and SOFA), whereas IL-1β and IL-10 levels had the strongest association with 30-day mortality. Lastly, patients with β-haemolytic streptococcal infection had higher levels of IL-6 and TNF-α compared with each subgroup stratified by microbial aetiology.

5.2 Strengths and limitations

Strengths

Several strengths should be emphasised. Firstly, the vast majority of studies investigating biomarkers in patients with NSTI are limited by a retrospective study design, which is particularly problematic in outcome assessment because the investigators are looking for patterns in data that are not specified a priori. In this thesis, we designed and successfully conducted three prospective, observational studies. The protocol of this thesis has been published and also registered at ClinicalTrials.gov, thus preventing data dredging and post-hoc revisions.

Secondly, we describe the results from the largest to date prospective, observational studies of patients with NSTI sampled with a follow-up of up to 2.7 years. In Scandinavia, we have a unique opportunity of patient follow-up due to our specific, assigned personal identification number (CPR-number) that can be linked to health databases (103). Therefore, this thesis provides valuable information about morbidity and mortality patterns, not only on a nationwide basis, but in the Scandinavian countries, which increases the external validity.

Thirdly, in Studies 1 and 2, we managed to include 98% of all patients transferred to Copenhagen University Hospital (Rigshospitalet), where the treatment of NSTI has been centralised. Moreover, the studies had only few inclusion and exclusion criteria. Both factors increase the likelihood of the study cohort being representative of the general population of patients with NSTI presented in the daily clinical settings. Unfortunately, in Study 3 we did not have data on how many patients with suspected NSTI from Sweden and Norway who were excluded because they showed not to have NSTI.

Further, the patients with NSTI represent a well-defined, homogenous population compared with patients in sepsis studies, which makes it easier and more reliable to study the dynamic course of the biomarkers and clinical implications.

Lastly, it is a strength that we managed to include all patients with dedicated teams consisting of only a small number of individuals, all using standardised sampling procedures, and that all clinical data were entered into a centralised online database by only a few individuals, thereby minimising the risk of information bias (104).

Limitations

A number of limitations need to be taken into consideration. Firstly, there might be a limitation to the external validity. In Studies 1 and 2, we observed that one in 10 patients did not have sepsis according to the classic definition (90). Some of the sickest patients may not have been transferred to our hospital due to haemodynamic instability or fatal outcome before transferring, thus increasing the risk of selection bias. However, we observed a mortality and amputation rate comparable to that of large retrospective studies (17,19,38,82,105,106).

Secondly, the studies might be subject to bias because of the inability to control for unknown confounders. As an example, high IL-6 expression is associated with increased age, female sex, current smoking, and advanced cancer (107–109). Differences in the NSTI groups according to these covariates might bias the results towards a higher or lower degree of association between the biomarker levels and the outcomes. We therefore chose to adjust for potential confounders (age, sex, chronic disease, SAPS II) that affect disease severity and mortality. The ideal way of controlling for unknown confounders is by conducting a randomised controlled trial. However, this type of study is not relevant when aiming to identify predictive risk factors and associations between biomarker levels and clinical outcomes, as was the intention of this thesis.

Thirdly, there is a limitation to the multivariate analysis model used in the studies. Even though we chose to adjust for the above-mentioned factors, there might be other relevant variables with an impact on outcome that are omitted, thus underfitting the model. However, it should be remembered that, as a rule of thumb, the number of explanatory variables should be limited to one tenth of the total number of outcome events to avoid overfitting when using logistic and Cox regression models (110–112). We tried to balance this knowledge with the most commonly used explanatory variables in sepsis biomarker studies. We are aware that age and some of the chronic diseases (malignancy and AIDS) are also included the SAPS II calculation. Importantly, we tested the validity of the a priori-planned regression model by removing age and chronic disease from the model (thus adjusting for only sex and SAPS II) without decreasing the precision of the regression coefficients. We therefore pragmatically chose to comply with our planned protocol.

Further, it is important to reflect on possible limitations of the missing SAPS II data. Only five patients (3.7%) had missing SAPS II values in Studies 1 and 2, whereas 31 patients (19.5%) had missing SAPS II values in Study 3. There are different statistical methods to assess the incomplete data, such as multiple imputation or substituting the missing values by the lowest and highest possible

value and then performing two analyses based on best/worst-case scenarios (113,114). We accounted for the incomplete data by describing the relevant baseline variables and outcomes of the patients with the missing SAPS II values, thereby providing transparency. This was done because SAPS II is a covariate in the adjusted analyses and not an outcome variable, and also because we had only few patients, predictors, and incidents of the outcome (mortality). Moreover, we performed the survival analyses by adjusting for the covariates in a stepwise manner, making it easier for the reader to assess the impact of the adjustments. Based on this, we do not believe that the statistical power of detecting relevant associations is reduced or that the comparability of the groups and the generalisability of the results are impaired. We acknowledge that there is no good way of dealing with missing data, but we have chosen this pragmatic approach. If the results are biased, it could be in any direction.

Additionally, we chose the Student t-test and the Mann-Whitney U-test for comparisons between groups, even though we measured multiple discrete time points in Studies 1 and 2. The Student t-test was used in order to quantify the difference between the groups and to see whether we risked overlooking a clinically relevant difference in biomarker levels, as defined in our sample size calculations. In Study 3, in particular, we might have overlooked a relevant difference as seen by the somewhat wide 95% CIs. The disadvantage of parametric tests is the assumption of normal distribution, which is rarely the case of any blood samples because of skewness. Data could be transformed using a logarithmic scale, but this often complicates interpretation of group differences and may confuse the reader. For significance testing, we therefore applied the Mann-Whitney U-test, which is a rank-sum test. In addition, it could be argued that it would have been interesting to analyse trends rather than difference in specific time points. However, if we had used the Kruskal-Wallis test, generalized linear mixed models, or area under the curve for the four discrete time points, we might have been able to provide an overall assessment, but it would be very difficult to apply this information to the clinical setting. If we had collected blood samples at more time points, it would have been relevant to include an analysis of trends over time to aid interpretation. We are aware that the analysis of differences at multiple time points will increase the risk of committing a type 1 error (rejecting the null hypothesis due to chance findings). This can be addressed by a multiple-comparison correction, such as the Bonferroni correction, but that will increase risk of making a type 2 error (overlooking a true difference). Therefore, we chose to focus on the baseline levels of the biomarkers. The baseline levels are the most relevant values for the clinicians, making risk stratification and guidance of treatment in the initial, acute phase of the disease, possible.

Furthermore, we were not able to establish a mechanistic explanation of the observed changes in the biomarker levels based on this study design. Thus, we do not know whether low Ficolin-2 level was due to primary deficiencies, consumption, or dilution (or all three combined). However, Ficolin-2 deficiency has not, to date, been reported (60). Unfortunately, we do not have albumin or haematocrit levels to estimate the degree of dilution. Nevertheless, Ficolin-2 has a higher molecular weight than PTX3 in plasma, making it unlikely that dilution plays a pivotal role in predicting disease outcome, especially as we found a high PTX3 level and low Ficolin-2 level to be associated with disease severity and mortality (94,115). A plausible explanation would be consumption of Ficolin-2, particularly in the light of Ficolin-2 being able to bind apoptotic cells and since Ficolin-2 is involved in the processing of nucleic acid released from necrotic cells (116). It could be argued that the extensive necrosis seen in patients with NSTI would result in Ficolin-2 overconsumption and a functional depletion state or that Ficolin-2 is a surrogate marker of tissue necrosis. Moreover, we do not know whether the high PTX3 level in patients with NSTI results in an uncontrolled amplification of the complement pathway and consumption of the ficolins. In line with this, it has been speculated whether the investigated plasma biomarker levels can be altered or removed by RRT and influence the results as 25% of our patients received RRT (117). This seems unlikely because PTX3 and ficolins have molecular weights around 400 kDa (62,66,94,115,118,119).

Finally, the studies were powered according to the primary analyses and to test the related hypotheses. Results from the secondary analyses need to be viewed with caution because a statistically significant association may represent a chance finding and insignificant results might represent underpowered studies. Nevertheless, these results can be used to provide information and generate hypotheses for future research. Additionally, we can only investigate the associations, but we are not able to conclude on causality, which is an inherent limitation to the observational studies. Thus, we do not know whether high levels of PTX3 and interleukins and low levels of Ficolins contribute to the deleterious inflammatory cascades and impairment of the immune responses or are simply results of the responses itself.

5.3 Current evidence and clinical implications

To the best of our knowledge, this thesis describes the largest prospective observational studies to date of patients with NSTI. In addition, no studies have investigated PTX3, ficolins, or MBL in this group of patients and only two small studies ($n < 20$) have prospectively examined the cytokine response (77,78).

Delay of surgery is an independent predictor of mortality in patients with NSTI, and studies stress the importance of multiple surgical debridements (17,20–22,120). However, an aggressive surgical approach increases the risk of severe disability and impaired quality of life. No standardised protocol for amputation as yet exists. We found that PTX3 was significantly higher in patients needing amputation. This was not the case for PCT and CRP. Therefore, PTX3 might be useful in identifying clinically relevant subgroups of patients with NSTI and should be taken into account to increase or decrease surgical aggressiveness. This needs to be addressed specifically in future studies. In addition, PCT measurements are expensive and with a new biomarker on the market, such as PTX3, which performs at least as well as PCT regarding disease severity and mortality, the hospital costs can potentially be reduced.

By investigating baseline PTX3 level, we now know that a high PTX3 level upon admission is related to poorer outcome. The same applies for low Ficolin-2 level upon admission and high baseline IL-1β and IL-10 levels. We believe this new knowledge is of value to the physicians at the emergency departments and in the ICUs. It is important to remember that the AUC for mortality prediction was relatively low for all of the investigated markers, which limits the clinical usefulness. Due to high negative predictive values and low positive predictive values, Ficolin-2 and the cytokines seem to be useful for ruling out patients in high risk of severe outcome rather than identifying high-risk patients (please refer to the manuscripts for the predictive values). In Study 1,

however, we found that the AUC for 180-day mortality could be increased by combining PTX3, PCT, and CRP. This suggests that a combination of biomarkers should be used rather than searching for one single effective marker. This is in line with the conclusion of a large review investigating prognostic biomarkers in patients with sepsis (50). A study using multiplex analysis also found that IL-1β and IL-6, amongst others, had good accuracy for predicting mortality before 48 hours, whereas IL-8 and monocyte chemoattractant protein-1 (MCP-1) had the best accuracy for predicting 28-day mortality (121). They also found that combining MCP-1 with the Acute Physiology and Chronic Health II score could markedly improve the diagnostic accuracy. A natural next step from here would therefore be to investigate IL-8 and MCP-1 and combine them with PTX3, Ficolin-2, Ficolin-3, IL-1β, IL-6, and IL-10, but also in combination with the SAPS II and SOFA scores.

It is difficult to translate the clinical condition of NSTI into a specific biomarker profile. However, the primary and secondary analyses were chosen with the clinical implications in mind. The presence of septic shock, a high LRINEC score, use of RRT, and amputation are associated with patient-important outcomes, including disability and mortality (42,43,122,123), making the results clinically relevant. We found that IL-1β, IL-6, IL-10 and TNF-α seem to have a place in the risk assessment of patients with NSTI and that these might improve the LRINEC score. Furthermore, we found variations in the cytokine responses in NSTIs depending on microbial aetiology, suggesting that different pathogenic mechanisms contribute to the disease. In particular, plasma levels of IL-6 and TNF-α might be used clinically to guide antibiotic treatment in patients with NSTI in cases where the microbiology is unknown. It has previously been shown that PCT and CRP levels can be used to guide treatment therapy and shorten antibiotic treatment duration in infected patients (124,125). In line with this, it is interesting that IL-6 and TNF-α levels were significantly increased in patients with β-haemolytic streptococcus infection as some streptococcal strains produce exotoxins, including superantigens, that result in excessive activation of the host response and release of proinflammatory cytokines, leading to septic shock, multiple organ failure, and death (23,126,127). It would be interesting to test whether immunoglobulin treatment could be undertaken based on guidance of IL-6 and/or TNF-α.

We adjusted for SAPS II in the analyses in order to find an independent marker of mortality and to make the results as clinically relevant as possible. It can be argued that the adjustment of SAPS II is a conservative approach because the score itself provides an estimate of the risk of death. By taking the SAPS II effect out of the comparison we might also remove the influence of some important pathophysiological processes that influence the disease severity and mortality. We therefore performed a stepwise adjustment to be able to determine the prognostic value of the biomarkers with and without the SAPS II adjustment. In addition, SAPS II calculation is based on 17 variables and it takes 24 hours before the physician can calculate the score. Accordingly, it is clinically relevant that we have identified PTX3, Ficolin-2, IL-1β, IL-6, IL-10, and TNF-α as important risk markers in patients with NSTI, also taking into account that SAPS II could not be calculated in almost 20% of the cases in Study 3. We also found that these biomarkers were closely correlated to the SAPS II.

5.4 Perspectives

An ideal biomarker reflects the biological and pathogenic responses, including responses to therapeutic interventions (107). More than 170 biomarkers have been investigated in patients with sepsis (50), all with limited specificity and sensitivity. Therefore, no biomarker has yet proved to be of great value to the clinicians. With this thesis we have identified biomarkers that are associated with clinically relevant outcomes in patients with NSTI. These biomarkers now need to be validated in other cohorts with this exact purpose and combined with disease severity scores in order to optimise the prognostic accuracy.

An interesting aspect of the innate immune response in the patients was the discovery of the high involvement of the ficolin-mediated lectin complement pathway compared with the classical pathway (Study 2). The only differences in activation of these two pathways are the PRMs (Ficolins and MBL vs. C1q, respectively) and the associated serine proteases (MASPs) (128), suggesting a particularly important role for the ficolins in infection control. As a spin-off of this new knowledge, it would be interesting to measure the plasma levels of MASPs in the patients to elucidate differences in pathway activation patterns.

With these studies we are one step closer to understanding some of the pathophysiological processes involved in the innate immune response, including the complement system, in patients with NSTI. However, important questions still need to be answered, especially regarding the diagnostic potential of the new biomarkers. In this thesis, we have investigated prognostic biomarkers in patients where the NSTI diagnosis is already confirmed. As a result, we have identified potential markers that might be relevant to elaborate on. However, logistical problems are involved in a study with the aim of investigating the diagnostic potential of biomarkers due to the rare nature of the disease. It would require a large scale setup to be able to include enough patients with suspected NSTI in the emergency departments. A next step could be to analyse the plasma samples from patients admitted with suspected NSTI, where the surgeons do not find any necrosis. These patients will have abscesses, erysipelas, and other non-necrotising skin infections and could function as a reference group. Furthermore, we need to identify the optimal discriminative cut-off value for each biomarker according to the relevant clinical outcomes. We used the median as a recurrent stratification value but there might be more sensitive and relevant levels to stratify by, as we demonstrated with Ficolin-2 in Study 2.

An ultimate future goal would be to individualise the treatment based on an objective set of biomarkers (129). In the three studies, we identified biomarkers that are useful in the risk stratification of patients with NSTI, thus getting us one step closer to making personalised medicine possible in this group of critically ill patients. Interestingly, studies have found that novel therapies, able to modify the pathophysiological process of sepsis, may also be guided by biomarkers (48,130,131). To our knowledge, only one randomised controlled trial has been conducted in patients with NSTI, investigating AB103, a novel synthetic CD28 mimetic peptide that selectively inhibits the direct binding of superantigen toxins (132). They found that high-dose AB103 could reduce plasma IL-6 and TNF-α level. Another study, which was terminated prematurely, found that one patient died in the group receiving immunoglobin compared with four in the placebo group (133). They also observed a significant decrease in the SOFA score on Day 2 and Day 3 in the treatment group. Combined with the knowledge obtained in Study 3, it might be relevant to conduct future studies with treatment directed specifically towards patients with high baseline levels of IL-6 and/or TNF-α in the hope of

being able to identify high-risk patients and initiate early treatment, thus preventing the devastating immunological responses and organ dysfunction. Another personalised strategy could be to use Ficolin-2 infusion as an adjuvant treatment in patients with low levels of Ficolin-2, as seen in phase-one clinical trials with MBL-infusion in MBL-deficient individuals (134–136). Finally, we plan to investigate whether hyperbaric oxygen therapy can decrease the inflammatory response by reducing the plasma level of IL-6 and other proinflammatory cytokines (87). If this is the case, then subgroups with high IL-6 level might particularly benefit from hyperbaric oxygen therapy. It might also be relevant with future studies randomising patients with a high inflammatory response to, for example, different surgical interventions, antibiotic treatment, or immunoglobulin therapy in order to determine the therapeutic effects in subgroups with a high risk of serious adverse events.

6. OVERALL CONCLUSION

These three studies collectively provide new knowledge on the aspects of the innate immune response in patients with NSTI. We have shown that patients with NSTI are characterised by a pronounced inflammatory response and that the response distinctively differs according to disease severity and mortality. Due to high negative predictive values, the biomarkers seem to be better suited at ruling out patients at high risk of severe outcome. However, all the biomarkers had a low AUC for mortality prediction, currently limiting the clinical value. Nevertheless, we have identified relevant biomarkers for disease severity and mortality. This knowledge can be used as guidance for future research in the field.

In patients with NSTI, PTX3 can be considered as a reliable marker of disease severity and mortality on equal terms with PCT. In addition, PTX3 might be able to identify clinically relevant subgroups, such as patients at risk of amputation. Baseline levels of Ficolin-2, Ficolin-3, and MBL below the median were significantly associated with short-term mortality, and Ficolin-2 was also associated with long-term mortality and correlated with SAPS II. Moreover, the ficolin-mediated lectin complement pathway seems to be the most implicated pathway in the pathogenic development in patients with NSTI. We found no significant association between the LRINEC score and cytokine levels on admission, suggesting that the LRINEC score does not reflect the cytokine response and inflammatory state in patients with NSTI. Baseline IL-6 had the strongest correlation with the disease severity scores (SAPS II and SOFA), whereas IL-1β and IL-10 had the strongest association with mortality. Finally, we found variations in the cytokine responses depending on microbial aetiology, suggesting that different pathogenic mechanisms contribute to the disease.

7. CONFLICTS OF INTEREST

None of the authors declares a conflict of interest.

8. SOURCE OF FUNDING

Major funding

European Union's Seventh Framework Programme
Rigshospitalet Research Foundation

Other funding

Aase and Ejnar Danielsen Foundation
Hans and Nora Buchard Foundation
Director Jacob Madsen and Olga Madsen Foundation
Christian Larsen and judge Ellen Larsen Foundation

9. SUMMARY OF THESIS

Necrotising soft tissue infection (NSTI) is a life-threatening and rapidly progressing bacterial infection involving one or more layers of the soft tissue compartments causing necrosis. The amputation and mortality rates remain high despite increased focus on the patients. Timely treatment, including surgical intervention, reduces the risk of severe disability and death. However, the lack of pathognomonic signs impedes early diagnosis and treatment. Moreover, the rarity of the disease makes it difficult to conduct large prospective studies, thus prospective research is almost non-existent in this group of patients. Instead data regarding biomarkers are extrapolated from the wide and heterogenic group of patients with sepsis, even though the immunological responses are likely to differ because of the large amount of necrotic tissue seen in patients with NSTI.

We performed the largest prospective, observational studies to date of patients with NSTI in Scandinavia sampled over more than two years with up to a 2.7-year follow-up. Blood samples were taken on admission (baseline) and the following three days and subsequently analysed for relevant plasma biomarkers. We elaborated on three aspects of the innate immune response, which included the investigation of acute-phase proteins, pattern recognition molecules of the lectin complement pathway, and inflammatory cytokines. The objective was to investigate aspects of the innate immune response in patients with NSTI, focusing on biomarkers as prognostic markers of disease severity and mortality. The overall hypothesis was that plasma biomarkers, representing the early innate immune response, can be used as prognostic markers of disease severity and mortality assessed by ICU scoring systems (SAPS II and SOFA score), the Laboratory Risk Indicator for Necrotizing Fasciitis (LRINEC) score, presence of septic shock, microbial aetiology, renal replacement therapy, and amputation.

In Study 1, we assessed the following acute-phase proteins in 135 patients with NSTI: pentraxin-3 (PTX3), procalcitonin, and C-reactive protein. We found that a high baseline PTX3 level above the median was significantly associated with the presence of septic shock, amputation, and 180-day mortality, albeit PTX3 was not an independent predictor of mortality. PTX3 and procalcitonin performed equally well, whereas C-reactive protein correlated poorly with clinically relevant outcomes.

In Study 2, we assessed the following plasma pattern recognition molecules in the same cohort as in Study 1: mannose-binding lectin, Ficolin-1, Ficolin-2, and Ficolin-3. We found that baseline Ficolin-2 level below the median was associated with short- and long-term mortality and correlated with the SAPS II, whereas low levels of mannose-binding lectin and Ficolin-3 were associated only with short-term mortality.

In Study 3, we assessed the following inflammatory cytokines in 159 patients with NSTI: interleukin-1β, interleukin-6, interleukin-10, and tumor necrosis factor-α. We found no significant association between the LRINEC score and baseline cytokine levels. In addition, interleukin-6 had the strongest correlation with the disease severity scores (SAPS II and SOFA score), whereas interleukin-1β and interleukin-10 had the strongest association with 30-day mortality. Moreover, patients with β-haemolytic

streptococcal infection had higher levels of interleukin-6 and tumor necrosis factor-α compared with each subgroup stratified by microbial aetiology.

This thesis provides new knowledge on the aspects of the innate immune response in patients with NSTI. The results prove that NSTI is characterised by a pronounced inflammatory response and that the innate immune response differs according to disease severity, microbial aetiology, and mortality. Through the three studies we have identified relevant biomarkers that are useful in the risk stratification of patients with NSTI, thus perhaps enhancing prognostication and decision making in these critically ill patients.

10. REFERENCES

1. Descamps V, Aitken J, Lee MG. Hippocrates on necrotising fasciitis. Lancet. 1994 Aug 20;344(8921):556.
2. Hasham S, Matteucci P, Stanley PRW, Hart NB. Necrotising fasciitis. BMJ. 2005 Apr 9;330(7495):830–3.
3. Sultan HY, Boyle AA, Sheppard N. Necrotising fasciitis. BMJ. 2012;345:e4274.
4. Giuliano A, Lewis F, Hadley K, Blaisdell FW. Bacteriology of necrotizing fasciitis. Am J Surg. 1977 Jul;134(1):52–7.
5. Morgan MS. Diagnosis and management of necrotising fasciitis: a multiparametric approach. J Hosp Infect. 2010 Aug;75(4):249–57.
6. Childers BJ, Potyondy LD, Nachreiner R, Rogers FR, Childers ER, Oberg KC, et al. Necrotizing fasciitis: a fourteen-year retrospective study of 163 consecutive patients. Am Surg. 2002 Feb;68(2):109–16.
7. Anaya DA, Dellinger EP. Necrotizing soft-tissue infection: diagnosis and management. Clin Infect Dis. 2007 Mar 1;44(5):705–10.
8. Hakkarainen TW, Kopari NM, Pham TN, Evans HL. Necrotizing soft tissue infections: review and current concepts in treatment, systems of care, and outcomes. Curr Probl Surg. 2014 Aug;51(8):344–62.
9. Hong G-L, Lu C-J, Lu Z-Q, Li M-F, Qiu Q-M, Liang H, et al. Surgical treatment of 19 cases with vibrio necrotising fasciitis. Burns. 2012 Mar;38(2):290–5.
10. Salcido RS. Necrotizing fasciitis: reviewing the causes and treatment strategies. Adv Skin Wound Care. 2007 May;20(5):288-293-295.
11. Das DK, Baker MG, Venugopal K. Increasing incidence of necrotizing fasciitis in New Zealand: a nationwide study over the period 1990 to 2006. J Infect. 2011 Dec;63(6):429–33.
12. Soltani AM, Best MJ, Francis CS, Allan BJ, Askari M, Panthaki ZJ. Trends in the incidence and treatment of necrotizing soft tissue infections: an analysis of the National Hospital Discharge Survey. J Burn Care Res. 2014 Oct;35(5):449–54.
13. Oud L, Watkins P. Contemporary trends of the epidemiology, clinical characteristics, and resource utilization of necrotizing fasciitis in Texas: a population-based cohort study. Crit Care Res Pract. 2015;2015:618067.
14. Ellis Simonsen SM, van Orman ER, Hatch BE, Jones SS, Gren LH, Hegmann KT, et al. Cellulitis incidence in a defined population. Epidemiol Infect. 2006 Apr;134(2):293–9.
15. Arif N, Yousfi S, Vinnard C. Deaths from necrotizing fasciitis in the United States, 2003-2013. Epidemiol Infect. 2015 Nov 9;1–7.
16. Luca-Harari B, Ekelund K, van der Linden M, Staum-Kaltoft M, Hammerum AM, Jasir A. Clinical and epidemiological aspects of invasive Streptococcus pyogenes infections in Denmark during 2003 and 2004. J Clin Microbiol. 2008 Jan;46(1):79–86.
17. Elliott DC, Kufera JA, Myers RA. Necrotizing soft tissue infections. Risk factors for mortality and strategies for management. Ann Surg. 1996 Nov;224(5):672–83.
18. Bosshardt TL, Henderson VJ, Organ CH. Necrotizing soft-tissue infections. Arch Surg. 1996 Aug;131(8):846-852-854.
19. Golger A, Ching S, Goldsmith CH, Pennie RA, Bain JR. Mortality in patients with necrotizing fasciitis. Plast Reconstr Surg. 2007 May;119(6):1803–7.
20. McHenry CR, Piotrowski JJ, Petrinic D, Malangoni MA. Determinants of mortality for necrotizing soft-tissue infections. Ann Surg. 1995 May;221(5):558–63.
21. Wong C-H, Chang H-C, Pasupathy S, Khin L-W, Tan J-L, Low C-O. Necrotizing fasciitis: clinical presentation, microbiology, and determinants of mortality. J Bone Joint Surg Am. 2003 Aug;85–A(8):1454–60.
22. Liu Y-M, Chi C-Y, Ho M-W, Chen C-M, Liao W-C, Ho C-M, et al. Microbiology and factors affecting mortality in necrotizing fasciitis. J Microbiol Immunol Infect. 2005 Dec;38(6):430–5.
23. Johansson L, Thulin P, Low DE, Norrby-Teglund A. Getting under the skin: the immunopathogenesis of Streptococcus pyogenes deep tissue infections. Clin Infect Dis. 2010 Jul 1;51(1):58–65.
24. Young MH, Aronoff DM, Engleberg NC. Necrotizing fasciitis: pathogenesis and treatment. Expert Rev Anti Infect Ther. 2005 Apr;3(2):279–94.
25. Davies HD, McGeer A, Schwartz B, Green K, Cann D, Simor AE, et al. Invasive group A streptococcal infections in Ontario, Canada. Ontario Group A Streptococcal Study Group. N Engl J Med. 1996 Aug 22;335(8):547–54.
26. Cunningham MW. Pathogenesis of group A streptococcal infections. Clin Microbiol Rev. 2000 Jul;13(3):470–511.
27. Bisno AL, Brito MO, Collins CM. Molecular basis of group A streptococcal virulence. Lancet Infect Dis. 2003 Apr;3(4):191–200.
28. Levine EG, Manders SM. Life-threatening necrotizing fasciitis. Clin Dermatol. 2005 Apr;23(2):144–7.
29. Nowak R. Flesh-eating bacteria: not new, but still worrisome. Science. 1994 Jun 17;264(5166):1665.
30. Sun X, Xie T. Management of Necrotizing Fasciitis and Its Surgical Aspects. Int J Low Extrem Wounds. 2015 Dec;14(4):328–34.

31. Wilkerson R, Paull W, Coville FV. Necrotizing fasciitis. Review of the literature and case report. Clin Orthop Relat Res. 1987 Mar;(216):187–92.

32. Leppard BJ, Seal DV. The value of bacteriology and serology in the diagnosis of necrotizing fasciitis. Br J Dermatol. 1983 Jul;109(1):37–44.

33. Canard B, Garnier T, Saint-Joanis B, Cole ST. Molecular genetic analysis of the nagH gene encoding a hyaluronidase of Clostridium perfringens. Mol Gen Genet. 1994 Apr;243(2):215–24.

34. Wong C-H, Wang Y-S. The diagnosis of necrotizing fasciitis. Curr Opin Infect Dis. 2005 Apr;18(2):101–6.

35. Sewell GS, Hsu VP, Jones SR. Zoster gangrenosum: necrotizing fasciitis as a complication of herpes zoster. Am J Med. 2000 Apr 15;108(6):520–1.

36. Trent JT, Kirsner RS. Diagnosing necrotizing fasciitis. Adv Skin Wound Care. 2002 Jun;15(3):135–8.

37. Singh G, Sinha SK, Adhikary S, Babu KS, Ray P, Khanna SK. Necrotising infections of soft tissues--a clinical profile. Eur J Surg. 2002;168(6):366–71.

38. Huang K-F, Hung M-H, Lin Y-S, Lu C-L, Liu C, Chen C-C, et al. Independent predictors of mortality for necrotizing fasciitis: a retrospective analysis in a single institution. J Trauma. 2011 Aug;71(2):467–473; discussion 473.

39. Goh T, Goh LG, Ang CH, Wong CH. Early diagnosis of necrotizing fasciitis. Br J Surg. 2014 Jan;101(1):e119-125.

40. Hussein QA, Anaya DA. Necrotizing soft tissue infections. Crit Care Clin. 2013 Oct;29(4):795–806.

41. Wong C-H, Khin L-W, Heng K-S, Tan K-C, Low C-O. The LRINEC (Laboratory Risk Indicator for Necrotizing Fasciitis) score: a tool for distinguishing necrotizing fasciitis from other soft tissue infections. Crit Care Med. 2004 Jul;32(7):1535–41.

42. Su Y-C, Chen H-W, Hong Y-C, Chen C-T, Hsiao C-T, Chen I-C. Laboratory risk indicator for necrotizing fasciitis score and the outcomes. ANZ J Surg. 2008 Nov;78(11):968–72.

43. Kincius M, Telksnys T, Trumbeckas D, Jievaltas M, Milonas D. Evaluation of LRINEC Scale Feasibility for Predicting Outcomes of Fournier Gangrene. Surg Infect (Larchmt). 2016 Mar 29;

44. Holland MJ. Application of the Laboratory Risk Indicator in Necrotising Fasciitis (LRINEC) score to patients in a tropical tertiary referral centre. Anaesth Intensive Care. 2009 Jul;37(4):588–92.

45. Tsai Y-H, Hsu RW-W, Huang K-C, Huang T-J. Laboratory indicators for early detection and surgical treatment of vibrio necrotizing fasciitis. Clin Orthop Relat Res. 2010 Aug;468(8):2230–7.

46. Thomas AJ, Meyer TK. Retrospective evaluation of laboratory-based diagnostic tools for cervical necrotizing fasciitis. Laryngoscope. 2012 Dec;122(12):2683–7.

47. Burner E, Henderson SO, Burke G, Nakashioya J, Hoffman JR. Inadequate Sensitivity of Laboratory Risk Indicator to Rule Out Necrotizing Fasciitis in the Emergency Department. West J Emerg Med. 2016 May;17(3):333–6.

48. Bernard GR, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med. 2001 Mar 8;344(10):699–709.

49. Mayr FB, Yende S, Linde-Zwirble WT, Peck-Palmer OM, Barnato AE, Weissfeld LA, et al. Infection rate and acute organ dysfunction risk as explanations for racial differences in severe sepsis. JAMA. 2010 Jun 23;303(24):2495–503.

50. Pierrakos C, Vincent J-L. Sepsis biomarkers: a review. Crit Care. 2010;14(1):R15.

51. Angus DC, van der Poll T. Severe sepsis and septic shock. N Engl J Med. 2013 Aug 29;369(9):840–51.

52. Mayr FB, Yende S, Angus DC. Epidemiology of severe sepsis. Virulence. 2014 Jan 1;5(1):4–11.

53. Beutler B. Innate immunity: an overview. Mol Immunol. 2004 Feb;40(12):845–59.

54. Medzhitov R, Janeway C. Innate immunity. N Engl J Med. 2000 Aug 3;343(5):338–44.

55. Janeway CA, Medzhitov R. Innate immune recognition. Annu Rev Immunol. 2002;20:197–216.

56. Lacy P, Stow JL. Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. Blood. 2011 Jul 7;118(1):9–18.

57. Walport MJ. Complement. First of two parts. N Engl J Med. 2001 Apr 5;344(14):1058–66.

58. Walport MJ. Complement. Second of two parts. N Engl J Med. 2001 Apr 12;344(15):1140–4.

59. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. Nat Rev Immunol. 2009 Oct;9(10):729–40.

60. Endo Y, Matsushita M, Fujita T. New insights into the role of ficolins in the lectin pathway of innate immunity. Int Rev Cell Mol Biol. 2015;316:49–110.

61. Holmskov U, Thiel S, Jensenius JC. Collections and ficolins: humoral lectins of the innate immune defense. Annu Rev Immunol. 2003;21:547–78.

62. Liu Y, Endo Y, Iwaki D, Nakata M, Matsushita M, Wada I, et al. Human M-ficolin is a secretory protein that activates the lectin complement pathway. J Immunol. 2005 Sep 1;175(5):3150–6.

63. Munthe-Fog L, Hummelshoj T, Honoré C, Moller ME, Skjoedt MO, Palsgaard I, et al. Variation in FCN1 affects biosynthesis of ficolin-1 and is associated with outcome of systemic inflammation. Genes Immun. 2012 Oct;13(7):515–22.

64. Matsushita M. Ficolins: complement-activating lectins involved in innate immunity. J Innate Immun. 2010;2(1):24–32.

65. Akaiwa M, Yae Y, Sugimoto R, Suzuki SO, Iwaki T, Izuhara K, et al. Hakata antigen, a new member of the ficolin/opsonin p35 family, is a novel human lectin secreted into

bronchus/alveolus and bile. J Histochem Cytochem. 1999 Jun;47(6):777–86.

66. Hummelshoj T, Fog LM, Madsen HO, Sim RB, Garred P. Comparative study of the human ficolins reveals unique features of Ficolin-3 (Hakata antigen). Mol Immunol. 2008 Mar;45(6):1623–32.

67. Eisen DP, Minchinton RM. Impact of mannose-binding lectin on susceptibility to infectious diseases. Clin Infect Dis. 2003 Dec 1;37(11):1496–505.

68. Gao D-N, Zhang Y, Ren Y-B, Kang J, Jiang L, Feng Z, et al. Relationship of serum mannose-binding lectin levels with the development of sepsis: a meta-analysis. Inflammation. 2015 Feb;38(1):338–47.

69. Chalmers JD, Fleming GB, Rutherford J, Matsushita M, Kilpatrick DC, Hill AT. Serum ficolin-2 in hospitalised patients with community-acquired pneumonia. Inflammation. 2014 Oct;37(5):1635–41.

70. Garlanda C, Bottazzi B, Bastone A, Mantovani A. Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. Annu Rev Immunol. 2005;23:337–66.

71. Bottazzi B, Doni A, Garlanda C, Mantovani A. An integrated view of humoral innate immunity: pentraxins as a paradigm. Annu Rev Immunol. 2010;28:157–83.

72. Mantovani A, Garlanda C, Doni A, Bottazzi B. Pentraxins in innate immunity: from C-reactive protein to the long pentraxin PTX3. J Clin Immunol. 2008 Jan;28(1):1–13.

73. Ma YJ, Doni A, Hummelshøj T, Honoré C, Bastone A, Mantovani A, et al. Synergy between ficolin-2 and pentraxin 3 boosts innate immune recognition and complement deposition. J Biol Chem. 2009 Oct 9;284(41):28263–75.

74. Ma YJ, Doni A, Skjoedt M-O, Honoré C, Arendrup M, Mantovani A, et al. Heterocomplexes of mannose-binding lectin and the pentraxins PTX3 or serum amyloid P component trigger cross-activation of the complement system. J Biol Chem. 2011 Feb 4;286(5):3405–17.

75. Doni A, Garlanda C, Bottazzi B, Meri S, Garred P, Mantovani A. Interactions of the humoral pattern recognition molecule PTX3 with the complement system. Immunobiology. 2012 Nov;217(11):1122–8.

76. Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature. 2007 Oct 18;449(7164):819–26.

77. Lungstras-Bufler K, Bufler P, Abdullah R, Rutherford C, Endres S, Abraham E, et al. High cytokine levels at admission are associated with fatal outcome in patients with necrotizing fasciitis. Eur Cytokine Netw. 2004 Jun;15(2):135–8.

78. Rodriguez RM, Abdullah R, Miller R, Barry L, Lungstras-Bufler K, Bufler P, et al. A pilot study of cytokine levels and white blood cell counts in the diagnosis of necrotizing fasciitis. Am J Emerg Med. 2006 Jan;24(1):58–61.

79. Moore SA, Levy BH, Prematilake C, Dissanaike S. The Prediction Predicament: Rethinking Necrotizing Soft Tissue Infections Mortality. Surg Infect (Larchmt). 2015 Aug 10;10.1089/sur.2015.002 [Epub ahead of print].

80. Al-Thani H, El-Menyar A, Shaikh N, Mudali IN, Mekkodathil A, Asim M, et al. Risk Stratification of Necrotizing Fasciitis Based on the Initial Procalcitonin Concentration: A Single Center Observational Study. Surg Infect (Larchmt). 2015 Dec;16(6):806–12.

81. Friederichs J, Hutter M, Hierholzer C, Novotny A, Friess H, Bühren V, et al. Procalcitonin ratio as a predictor of successful surgical treatment of severe necrotizing soft tissue infections. Am J Surg. 2013 Sep;206(3):368–73.

82. Anaya DA, McMahon K, Nathens AB, Sullivan SR, Foy H, Bulger E. Predictors of mortality and limb loss in necrotizing soft tissue infections. Arch Surg. 2005 Feb;140(2):151–157; discussion 158.

83. Yaghoubian A, de Virgilio C, Dauphine C, Lewis RJ, Lin M. Use of admission serum lactate and sodium levels to predict mortality in necrotizing soft-tissue infections. Arch Surg. 2007 Sep;142(9):840–6.

84. Schwartz S, Kightlinger E, de Virgilio C, de Virgilio M, Kaji A, Neville A, et al. Predictors of mortality and limb loss in necrotizing soft tissue infections. Am Surg. 2013 Oct;79(10):1102–5.

85. Krieg A, Dizdar L, Verde PE, Knoefel WT. Predictors of mortality for necrotizing soft-tissue infections: a retrospective analysis of 64 cases. Langenbecks Arch Surg. 2014 Mar;399(3):333–41.

86. Keeley J, Kaji A, Kim D, Yan H, Putnam BA, Plurad D, et al. Predictors of mortality in necrotizing soft tissue infection. Am Surg. 2014 Oct;80(10):989–93.

87. Hansen MB, Simonsen U, Garred P, Hyldegaard O. Biomarkers of necrotising soft tissue infections: aspects of the innate immune response and effects of hyperbaric oxygenation-the protocol of the prospective cohort BIONEC study. BMJ Open. 2015;5(5):e006995.

88. Le Gall JR, Lemeshow S, Saulnier F. A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study. JAMA. 1993 Dec 22;270(24):2957–63.

89. Vincent JL, Moreno R, Takala J, Willatts S, De Mendonça A, Bruining H, et al. The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. Intensive Care Med. 1996 Jul;22(7):707–10.

90. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. Chest. 1992 Jun;101(6):1644–55.

91. Leng SX, McElhaney JE, Walston JD, Xie D, Fedarko NS, Kuchel GA. ELISA and multiplex technologies for cytokine measurement in inflammation and aging research. J Gerontol A Biol Sci Med Sci. 2008 Aug;63(8):879–84.

92. Bastrup-Birk S, Skjoedt M-O, Munthe-Fog L, Strom JJ, Ma YJ, Garred P. Pentraxin-3 serum levels are associated with disease severity and mortality in patients with systemic inflammatory response syndrome. PLoS ONE. 2013;8(9):e73119.

93. Bastrup-Birk S, Munthe-Fog L, Skjoedt M-O, Ma YJ, Nielsen H, Køber L, et al. Pentraxin-3 level at admission is a strong predictor of short-term mortality in a community-based hospital setting. J Intern Med. 2015 May;277(5):562–72.

94. Munthe-Fog L, Hummelshøj T, Hansen BE, Koch C, Madsen HO, Skjødt K, et al. The impact of FCN2 polymorphisms and haplotypes on the Ficolin-2 serum levels. Scand J Immunol. 2007 Apr;65(4):383–92.

95. Munthe-Fog L, Hummelshøj T, Ma YJ, Hansen BE, Koch C, Madsen HO, et al. Characterization of a polymorphism in the coding sequence of FCN3 resulting in a Ficolin-3 (Hakata antigen) deficiency state. Mol Immunol. 2008 May;45(9):2660–6.

96. Garred P, Madsen HO, Kurtzhals JA, Lamm LU, Thiel S, Hey AS, et al. Diallelic polymorphism may explain variations of the blood concentration of mannan-binding protein in Eskimos, but not in black Africans. Eur J Immunogenet. 1992 Dec;19(6):403–12.

97. Seelen MA, Roos A, Wieslander J, Mollnes TE, Sjöholm AG, Wurzner R, et al. Functional analysis of the classical, alternative, and MBL pathways of the complement system: standardization and validation of a simple ELISA. J Immunol Methods. 2005 Jan;296(1–2):187–98.

98. Hein E, Honoré C, Skjoedt M-O, Munthe-Fog L, Hummelshøj T, Garred P. Functional analysis of Ficolin-3 mediated complement activation. PLoS ONE. 2010;5(11):e15443.

99. Muller B, Peri G, Doni A, Torri V, Landmann R, Bottazzi B, et al. Circulating levels of the long pentraxin PTX3 correlate with severity of infection in critically ill patients. Crit Care Med. 2001 Jul;29(7):1404–7.

100. Mauri T, Bellani G, Patroniti N, Coppadoro A, Peri G, Cuccovillo I, et al. Persisting high levels of plasma pentraxin 3 over the first days after severe sepsis and septic shock onset are associated with mortality. Intensive Care Med. 2010 Apr;36(4):621–9.

101. Harbarth S, Holeckova K, Froidevaux C, Pittet D, Ricou B, Grau GE, et al. Diagnostic value of procalcitonin, interleukin-6, and interleukin-8 in critically ill patients admitted with suspected sepsis. Am J Respir Crit Care Med. 2001 Aug 1;164(3):396–402.

102. Behnes M, Bertsch T, Lepiorz D, Lang S, Trinkmann F, Brueckmann M, et al. Diagnostic and prognostic utility of soluble CD 14 subtype (presepsin) for severe sepsis and septic shock during the first week of intensive care treatment. Crit Care. 2014;18(5):507.

103. Frank L. Epidemiology. When an entire country is a cohort. Science. 2000 Mar 31;287(5462):2398–9.

104. Althubaiti A. Information bias in health research: definition, pitfalls, and adjustment methods. J Multidiscip Healthc. 2016;9:211–7.

105. Frazee BW, Fee C, Lynn J, Wang R, Bostrom A, Hargis C, et al. Community-acquired necrotizing soft tissue infections: a review of 122 cases presenting to a single emergency department over 12 years. J Emerg Med. 2008 Feb;34(2):139–46.

106. Dworkin MS, Westercamp MD, Park L, McIntyre A. The epidemiology of necrotizing fasciitis including factors associated with death and amputation. Epidemiol Infect. 2009 Nov;137(11):1609–14.

107. Ershler WB, Keller ET. Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. Annu Rev Med. 2000;51:245–70.

108. Duffy SA, Taylor JMG, Terrell JE, Islam M, Li Y, Fowler KE, et al. Interleukin-6 predicts recurrence and survival among head and neck cancer patients. Cancer. 2008 Aug 15;113(4):750–7.

109. Chen C-J, Sung W-W, Lin Y-M, Chen M-K, Lee C-H, Lee H, et al. Gender difference in the prognostic role of interleukin 6 in oral squamous cell carcinoma. PLoS ONE. 2012;7(11):e50104.

110. Concato J, Peduzzi P, Holford TR, Feinstein AR. Importance of events per independent variable in proportional hazards analysis. I. Background, goals, and general strategy. J Clin Epidemiol. 1995 Dec;48(12):1495–501.

111. Peduzzi P, Concato J, Feinstein AR, Holford TR. Importance of events per independent variable in proportional hazards regression analysis. II. Accuracy and precision of regression estimates. J Clin Epidemiol. 1995 Dec;48(12):1503–10.

112. Peduzzi P, Concato J, Kemper E, Holford TR, Feinstein AR. A simulation study of the number of events per variable in logistic regression analysis. J Clin Epidemiol. 1996 Dec;49(12):1373–9.

113. Marshall A, Altman DG, Holder RL. Comparison of imputation methods for handling missing covariate data when fitting a Cox proportional hazards model: a resampling study. BMC Med Res Methodol. 2010;10:112.

114. Johansson ÅM, Karlsson MO. Comparison of methods for handling missing covariate data. AAPS J. 2013 Oct;15(4):1232–41.

115. Inforzato A, Jaillon S, Moalli F, Barbati E, Bonavita E, Bottazzi B, et al. The long pentraxin PTX3 at the crossroads between innate immunity and tissue remodelling. Tissue Antigens. 2011 Apr 1;77(4):271–82.

116. Kuraya M, Ming Z, Liu X, Matsushita M, Fujita T. Specific binding of L-ficolin and H-ficolin to apoptotic cells leads to complement activation. Immunobiology. 2005;209(9):689–97.

117. Honore PM, Spapen HD. Pentraxin-3 to better delineate necrotizing soft tissue infection: not really! Crit Care. 2016;20(1):173.

118. Kilpatrick DC, Chalmers JD. Human L-ficolin (ficolin-2) and its clinical significance. J Biomed Biotechnol. 2012;2012:138797.

119. Hummelshoj T, Thielens NM, Madsen HO, Arlaud GJ, Sim RB, Garred P. Molecular organization of human Ficolin-2. Mol Immunol. 2007 Jan;44(4):401–11.

120. Bilton BD, Zibari GB, McMillan RW, Aultman DF, Dunn G, McDonald JC. Aggressive surgical management of necrotizing fasciitis serves to decrease mortality: a retrospective study. Am Surg. 1998 May;64(5):397–400.

121. Bozza FA, Salluh JI, Japiassu AM, Soares M, Assis EF, Gomes RN, et al. Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis. Crit Care. 2007;11(2):R49.

122. Laupland KB, Zygun DA, Doig CJ, Bagshaw SM, Svenson LW, Fick GH. One-year mortality of bloodstream infection-associated sepsis and septic shock among patients presenting to a regional critical care system. Intensive Care Med. 2005 Feb;31(2):213–9.

123. Bagshaw SM, Uchino S, Bellomo R, Morimatsu H, Morgera S, Schetz M, et al. Septic acute kidney injury in critically ill patients: clinical characteristics and outcomes. Clin J Am Soc Nephrol. 2007 May;2(3):431–9.

124. Schuetz P, Müller B, Christ-Crain M, Stolz D, Tamm M, Bouadma L, et al. Procalcitonin to initiate or discontinue antibiotics in acute respiratory tract infections. Cochrane Database Syst Rev. 2012;(9):CD007498.

125. Oliveira CF, Botoni FA, Oliveira CRA, Silva CB, Pereira HA, Serufo JC, et al. Procalcitonin versus C-reactive protein for guiding antibiotic therapy in sepsis: a randomized trial. Crit Care Med. 2013 Oct;41(10):2336–43.

126. Kotb M. Bacterial pyrogenic exotoxins as superantigens. Clin Microbiol Rev. 1995 Jul;8(3):411–26.

127. Norrby-Teglund A, Thulin P, Gan BS, Kotb M, McGeer A, Andersson J, et al. Evidence for superantigen involvement in severe group a streptococcal tissue infections. J Infect Dis. 2001 Oct 1;184(7):853–60.

128. Charchaflieh J, Rushbrook J, Worah S, Zhang M. Activated Complement Factors as Disease Markers for Sepsis. Dis Markers. 2015;2015:382463.

129. Willis JCD, Lord GM. Immune biomarkers: the promises and pitfalls of personalized medicine. Nat Rev Immunol. 2015 May;15(5):323–9.

130. Dhainaut J-F, Laterre P-F, Janes JM, Bernard GR, Artigas A, Bakker J, et al. Drotrecogin alfa (activated) in the treatment of severe sepsis patients with multiple-organ dysfunction: data from the PROWESS trial. Intensive Care Med. 2003 Jun;29(6):894–903.

131. Panacek EA, Marshall JC, Albertson TE, Johnson DH, Johnson S, MacArthur RD, et al. Efficacy and safety of the monoclonal anti-tumor necrosis factor antibody F(ab')2 fragment afelimomab in patients with severe sepsis and elevated interleukin-6 levels. Crit Care Med. 2004 Nov;32(11):2173–82.

132. Bulger EM, Maier RV, Sperry J, Joshi M, Henry S, Moore FA, et al. A Novel Drug for Treatment of Necrotizing Soft-Tissue Infections: A Randomized Clinical Trial. JAMA Surg. 2014 Jun;149(6):528–36.

133. Darenberg J, Ihendyane N, Sjölin J, Aufwerber E, Haidl S, Follin P, et al. Intravenous immunoglobulin G therapy in streptococcal toxic shock syndrome: a European randomized, double-blind, placebo-controlled trial. Clin Infect Dis. 2003 Aug 1;37(3):333–40.

134. Valdimarsson H. Infusion of plasma-derived mannan-binding lectin (MBL) into MBL-deficient humans. Biochem Soc Trans. 2003 Aug;31(Pt 4):768–9.

135. Valdimarsson H, Vikingsdottir T, Bang P, Saevarsdottir S, Gudjonsson JE, Oskarsson O, et al. Human plasma-derived mannose-binding lectin: a phase I safety and pharmacokinetic study. Scand J Immunol. 2004 Jan;59(1):97–102.

136. Bang P, Laursen I, Thornberg K, Schierbeck J, Nielsen B, Valdimarsson H, et al. The pharmacokinetic profile of plasma-derived mannan-binding lectin in healthy adult volunteers and patients with Staphylococcus aureus septicaemia. Scand J Infect Dis. 2008;40(1):44–8.