New Markers for Infection and New Methods in Evaluation of the Inflammatory Status in Patients with Sepsis

Ph.D. Thesis

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PREFACE

This Ph.D. thesis is based on five manuscripts. The project was conducted as a Ph.D. study at the University of Southern Denmark in the period 2003-2006. The last manuscripts were written in the period 2006-2007.

My interest in inflammation and severe acute infections was raised during a clinical stay as a medical student in 1995 at the Trauma Department at Roosevelt Medical Centre, NYC. Trauma surgeon Alain Corcos introduced me to the systemic inflammatory response syndrome and the pro-inflammatory aspects in sepsis and trauma. An inspiring clinical stay in 1996 at the Department of Infectious Diseases at Ullevål University Hospital, Oslo, introduced me to the exciting field of tropical and infectious diseases. A pre-graduate research project in 1997 looking upon aspects of the innate immune response in bacterial meningitis under skilled supervision by Professor Jens D. Lundgren, Department of Infectious Diseases, Hvidovre University Hospital, Copenhagen, prepared me for a post-graduate research project. After five years of clinical work, I started an exciting journey as a Ph.D. student "navigating" my own "research ship". Associate Professor Svend S. Pedersen and Professor Court Pedersen, both from the Department of Infectious Diseases, Odense University Hospital, welcomed me in their research group despite that I had no "previous history" in Odense, with an educational background from the University of Copenhagen. They believed in me, supported me and encouraged me in the first difficult phases of the project. They were patient in their supervision despite my stubbornness. I have benefited very much from their sound scientific advises and vast clinical experience. The participation in the project by Associate Professor Ole G. Koldkjær, Consultant Holger J. Møller and Professor Søren K. Moestrup was crucial for the laboratory parts of the project. I met these excellent researchers by coincident and benefited very much from our cooperation. They contributed with their state of the art theoretical and clinical knowledge within the field of biochemistry. Thanks to Professor Bente Klarlund Pedersen and Virologist Lars P. Nielsen for their support. Thanks to Virologist Lars P. Nielsen for inspiring hour long academic discussions when I was affiliated to the Department of Clinical Microbiology. Without our excellent study nurses Lene Hergens, Nete Bülow, Anita Nymark and Helle Møller the great task of including so many patients with infections and sepsis would have been impossible. Thanks to you! Thanks to all competent clinical nurses at the Medical Department C, Odense University Hospital! Thanks to the competent laboratory technicians who draw and processed thousands of research blood samples over several years! Thanks to the competent doctors at the Department of Clinical Microbiology, Odense University Hospital, who thoroughly identified pathogens from clinical samples and called me whenever they identified a patient with bacteremia in a period of almost two years! Thanks to the competent research laboratory technicians Joan Clausen, Hanne Madsen and Kirsten B. Petersen who analyzed thousands of research samples in a thorough and systematic manner! Thanks to fellow Ph.D. students at Odense University Hospital, Hvidovre University Hospital and Skejby University Hospital for fruitful discussions both in academia and in more relaxed conditions! Thanks to all public and private foundations who generously supported my study: University of Southern Denmark, Toyota Foundation in Denmark, M.L. Jørgensen and Gunnar Hansen Foundation, Research Foundation of the Danish Medical Association, H. Christensen Foundation, K. and V. Skovgaards Foundation, J. and O. Madsens Foundation, J. Boserup Foundation, Odense University Hospital Consultant Foundation, Foundation of Medical Research in the County of Fyn, C. Larsen and Judge E. Larsens Foundation, P.A. Messerschmidt and Wife foundation, County of Southern Jutland Research Foundation.

Thanks to my father Engineer Hossein Gaïni and my mother High school teacher Lív Joensen who always have encouraged me to make my own decisions on my life path regarding education, ambitions and my personal life. Thanks to my Faroese grand father Lawyer, Author and Politician the late Sigurð Joensen for always supporting me and urging me to strive for excellence. Thanks to my brother Social Anthropologist Ph.D. Firouz Gaïni and my sister Poet and Actress Sigri Gaïni for always believing in my life projects and supporting me on my life path. Thanks to my best friend in Norway Axel Lindemann for always reminding me that fly fishing for salmon and trout are more important than trivialities like work, career and research. Thanks for your friendship! Thanks to my best Faroese friends Sølvi Joensen, Brian Joensen and Brynleif Hansen for our friendship since early childhood! Thanks to my friend Mette Nørlyng for always having supported my ambitions, projects and always believed in me! Thanks to all other friends and family for you support, love and friendship!

Finally I want to thank my precious, beautiful and bright children Laura Marie and Viktor Johan for your love! You are always asking the right questions and you have the amazing curiosity in all aspects of life often lost in adult life! Keep on with that!

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Paper 2:

Gaini S, Koldkjaer OG, Pedersen C, Pedersen SS. Procalcitonin, lipopolysaccharide-binding protein, interleukin-6 and C-reactive protein in community-acquired infections and sepsis: a prospective study. Critical Care. 2006;10(2):R53.

Paper 3:

Gaini S, Koldkjaer OG, Pedersen SS, Pedersen C, Moestrup SK, Moller HJ. Soluble haemoglobin scavenger receptor (sCD163) in patients with suspected community-acquired infections. APMIS. 2006 Feb;114(2):103-11

Paper 4:

Gaini S, Pedersen SS, Pedersen C, Koldkjaer OG, Moller HJ. High mobility group box-1 protein (HMGB1) in patients with suspected community-acquired infections and sepsis: a prospective study. Critical Care. 2007 Mar 8;11(2):R32

Paper 5:

Gaini S, Pedersen SS, Koldkjaer OG, Pedersen C, Moestrup SK, Moller HJ. New immunological markers in bacteremia: Anti-inflammatory soluble CD163, but not pro-inflammatory HMGB1, is related to prognosis (submitted: Clinical and Experimental Immunology)

II. ABBREVIATIONS

SIRS	Systemic inflammatory response syndrome
IL-4	Interleukin-4
IL-10	Interleukin-10
PRR	Pathogen recognition receptor
PAMP	Pathogen-associated molecular pattern
TLR	Toll-like receptor
LBP	Lipopolysaccharide-binding protein
IL-1	Interleukin-1
TNF-a	Tumour necrosis factor-alpha
IL-6	Interleukin-6
HMGB1	High mobility group box-1 protein
LPS	Lipopolysaccharide
CARS	Compensatory anti-inflammatory response syndrome
HLA-DR	Major histocompatibility complex
aa	Amino acids
sCD163	Soluble haemoglobin scavenger receptor
CRP	C-reactive protein
WBC	White blood cell
APP	Acute phase protein
PCT	Procalcitonin
AM	Adrenomedullin
SOFA	Sequential organ failure assessment score
APACHE	Acute physiology and chronic health evaluation score
SRCR	Scavenger receptor cystein-rich family
sTREM1	Soluble triggering receptor expressed on myeloid cells-1

III. English summary

Sepsis is a serious condition with a high morbidity and mortality affecting an increasing number of patients. There is a need of developing new diagnostic modalities for identifying infected patients with and without the systemic inflammatory response syndrome. The biochemical tests used in clinical routine today are characterized by reasonable sensitivities but low specificities. Several new markers have during the last years been suggested as possible markers of infection and sepsis in the clinical setting. During the last years there has also been an increased attention on the anti-inflammatory aspects in serious infections. The introduction of the SIRS criteria were based upon a paradigm focusing on the pro-inflammation in sepsis. Many animal and human trials focused upon blockage of different pro-inflammatory pathways in sepsis. In general the human studies were disappointing. During the last few years a new paradigm in sepsis has focused on pro-inflammation and anti-inflammation either consecutively or in parallel in different focus/body compartments. This has urged research to look for new methods to evaluate the immune status of each individual sepsis patient. An effective way of assessing the immune status of the individual sepsis patient could maybe be followed by specific immuno-modulating therapy on the individual level.

The aims of this study was ***a.*** to evaluate several new candidate molecules for diagnosing infection and sepsis and ***b.*** to evaluate levels of several newly described pro-and anti-inflammatory molecules in infection and sepsis. The study design was a prospective observational clinical study including patients in the milder end of the sepsis spectrum in 3 cohorts from a department of internal medicine at a Danish university hospital. The 3 cohorts covered the entire spectrum of infection and sepsis at a department of internal medicine. Patients were sampled and plasma/serum/whole blood were stored until laboratory analyzes were performed in batches.

Cohort A.1: One hundred and eighty-five adult patients suspected of having severe community-acquired sepsis were included in a prospective manner over a 23 months period. These patients were sampled on a daily basis in up to five days depending on their outcome or discharge time. Levels of HMGB1, PCT, LBP, IL-6, CRP, WBC and neutrophils were measured. The aim of this study was to evaluate levels of the pro-inflammatory cytokine HMGB1, the infection marker PCT and the acute phase protein LBP in infection/sepsis of different severity. The diagnostic test abilities of HMGB1, PCT and LBP in diagnosing the presence of bacteremia were also evaluated.

Cohort A.2: One hundred and ninety-four adult patients suspected of having community-acquired infections were included in a prospective manner over a 5 months period. These patients were sampled once immediately after admission to hospital. Levels of HMGB1, sCD163, PCT, LBP, IL-6, CRP, WBC and neutrophils were measured. The aim of this study was to evaluate PCT, LBP, IL-6 and CRP as diagnostic test markers for infection and sepsis in patients admitted to a department of internal medicine. Another aim was to evaluate the pro-inflammatory cytokine HMGB1 and sCD163 as molecular markers in mild infections and sepsis.

Cohort B: One hundred and ten patients admitted to a department of internal medicine with microbiologically verified bacteremia were included in a prospective manner in a 19 months period. These patients were sampled on a daily basis in up to five days depending on their outcome or discharge time. Levels of HMGB1, sCD163, PCT, LBP, IL-6, IL-10, CRP, WBC and neutrophils were measured. The aim of this study was to evaluate the pro-inflammatory cytokine HMGB1 and sCD163 as immunological and prognostic markers in patients with a robust gold-standard for the presence of infection.

In conclusion our data do not suggest that PCT should be introduced as a routine test in diagnosing infection and sepsis in patients with suspected community acquired mild infections/sepsis admitted to a department of internal medicine. PCT and LBP are severity markers in sepsis and PCT is a marker for the presence of bacteremia. LBP and IL-6 seem to perform equally to CRP as diagnostic test markers for community-acquired mild infections/sepsis in patients admitted to a department of internal medicine. LBP did not discriminate between gram-negative and gram-positive bacteremia. sCD163 and HMGB1 did not discriminate between non-infected patients and infected patients. Levels of sCD163 were only elevated in severe sepsis and bacteremia. sCD163 and IL-6 were prognostic markers in patients with bacteremia. sCD163 correlated to the measured anti-inflammatory markers suggesting an anti-inflammatory role for sCD163. Levels of HMGB1 were elevated in infected patients compared to healthy controls. HMGB1 correlated to the measured pro-inflammatory markers suggesting a pro-inflammatory role for this cytokine.

There is still a need to continue efforts in identifying new possible candidates for diagnostic biochemical markers in infection and sepsis. These should have higher sensitivities and especially higher specificities than the markers used in clinical routine today. Increased insight in the

immunopathogenesis of sepsis would offer the potential to generate new diagnostic and treatment options in sepsis.

IV. Danish summary

Sepsis (blodforgiftning) er en alvorlig tilstand med høj dødelighed og sygelighed som rammer et stigende antal patienter. Der er et behov for at udvikle nye diagnostiske og prognostiske metoder til at identificere inficerede patienter med og uden systemisk inflammatorisk respons syndrom. De biokemiske prøver man råder over i den daglige klinik i dag er kendetegnet ved rimelig sensitivitet og lav specificitet. Flere nye markører er for nylig blevet foreslået som mulige markører for infektion og sepsis. Gennem de seneste år er der kommet øget opmærksomhed på antiinflammatoriske aspekter ved sepsis. Introduktionen af begrebet systemisk inflammatorisk respons syndrom var baseret på et paradigme fokuserende på proinflammatoriske aspekter ved sepsis. Mange dyreforsøg og kliniske studier fokuserende på hæmning af forskellige proinflammatoriske systemer ved sepsis viste skuffende resultater. Gennem de seneste årene er et nyt paradigme ved sepsis introduceret fokuserende både på proinflammation og antiinflammation enten konsekutivt eller parallelt i forskellige infektionsfokus og i forskellige deler af kroppen. Dette paradigme har peget mod et behov for udvikling af nye metoder hvor man kan vurdere den enkelte sepsis patients immuntilstand. En effektiv målemetode for immunstatus i den enkelte sepsis patient ville muligvis kunne blive fulgt af målrettet immunmodulerende behandling til den enkelte patient.

Formålene med dette studium var ***a.*** at undersøge flere nye diagnostiske kandidat molekyler ved infektion og sepsis og ***b.*** at undersøge flere nylig beskrevne pro-og antiinflammatoriske molekyler ved infektion og sepsis. Studiedesignet var et prospektivt observationelt klinisk studium hvor man inkluderede patienter i den milde ende af sepsis i tre kohorter fra en intern medicinsk afdeling på et dansk universitetshospital. Disse tre kohorter dækkede hele spektret af infektion og sepsis på en intern medicinsk afdeling. Der blev taget blodprøver fra patienterne og blod blev frosset indtil laboratorieanalyserne blev gennemført i forskellige forskningslaboratorier.

Kohorte A.1: 185 voksne patienter mistænkt for at have alvorlige samfundserhvervet sepsis blev inkluderede prospektivt over en 23 måneders periode. Disse patienter fik taget blodprøver dagligt i op til fem dage eller indtil udskrivning eller død. Niveauerne af HMGB1, PCT, LBP, IL-6, CRP, leukocytter og neutrofile blev målt. Formålet med dette studium var at undersøge niveauerne af det proinflammatoriske cytokin HMGB1, infektionsmarkøren PCT og akut fase proteinet LBP ved infektion/sepsis med forskellig sværhedsgrad. De diagnostiske test egenskaber til HMGB1, PCT og LBP i at diagnosticere bakteriæmi blev også undersøgt.

Kohorte A.2: 194 voksne patienter mistænkt for at have samfundserhvervede infektioner blev inkluderet prospektivt over en 5 måneders periode. Disse patienter fik taget en enkelt blodprøve umiddelbart efter indlæggelse. Niveauerne af HMGB1, sCD163, PCT, LBP, IL-6, CRP, leukocytter og neutrofile blev målt. Formålet med dette studium var at undersøge de diagnostiske test egenskaber til PCT, LBP, IL-6 og CRP i at diagnosticere infektion og sepsis hos patienter indlagt på en intern medicinsk afdeling. Et andet formål var at undersøge det proinflammatoriske cytokin HMGB1 og sCD163 som molekylære markører ved milde infektioner og sepsis.

Kohorte B: 110 patienter indlagt på en intern medicinsk afdeling med verificeret bakteriæmi blev inkluderet prospektivt over en 19 måneders periode. Disse patienter fik taget blodprøver dagligt i op til fem dage eller indtil udskrivning eller død. Niveauerne af HMGB1, sCD163, PCT, LBP, IL-6, IL-10, CRP, leukocytter og neutrofile blev målt. Formålet med dette studium var at undersøge det proinflammatoriske cytokin HMGB1 og sCD163 som immunologiske og prognostiske markører hos patienter med en sikker guldstandard for tilstedeværelsen af infektion.

Studiets data tyder ikke på at PCT skal introduceres som diagnostisk rutinetest for infektion og sepsis hos patienter med mistænkt samfundserhvervede milde infektioner/sepsis indlagt på intern medicinske afdelinger. PCT og LBP er markører for sværhedsgraden af sepsis og PCT er en markør for tilstedeværelsen af bakteriæmi. LBP og IL-6 ser ud til at være ligeværdige med CRP som diagnostiske test markører for samfundserhvervede milde infektioner/sepsis hos patienter indlagt på intern medicinske afdelinger. LBP skelner ikke mellem gram-positiv og gram-negativ bakteriæmi. sCD163 og HMGB1 kan ikke skelne mellem ikke inficerede og inficerede patienter. Niveauerne af sCD163 var kun øget hos patienter med svær sepsis og hos patienter med bakteriæmi. sCD163 korrelerede til de målte antiinflammatoriske markører tydende på en antiinflammatorisk rolle for sCD163. Niveauerne af HMGB1 var øget hos inficerede patienter sammenlignet med raske kontroller. HMGB1 korrelerede til de målte proinflammatoriske markører tydende på en proinflammatorisk rolle for dette cytokin.

Der er fortsat behov for videre indsats hvad angår forsøg på at identificere nye mulige diagnostiske test markører for infektion og sepsis. Disse bør have højere sensitivitet og især højere specificitet sammenlignet med de tests der bruges i den daglige rutine på nuværende tidspunkt. Øget indsigt i

immunpatogenesen ved sepsis vil kunne åbne for nye diagnostiske og terapeutiske muligheder ved sepsis.

V. BACKGROUND

V.1 Sepsis

The clinical syndrome of sepsis has been known since ancient time. The Greek word *sepsis* means putrefaction [1]. This syndrome was well known by physicians and surgeons. They often saw this syndrome in wounded patients and in women giving birth. The last mentioned group feared the puerperal sepsis. A major breakthrough in prevention of this often fatal condition was the introduction of hand hygiene in connection to birth by the Hungarian-Austrian doctor I.P. Semmelweis [2]. Another group of patients often affected by sepsis were wounded soldiers with blunt and penetrating traumas. Even if these wounded survived the blood loss, they often succumbed due to infectious wound complications and sepsis [3]. The Danish physiologist P.L. Panum conducted several animal experiments in 1853 at the University of Kiel where he identified a substance that could induce shock in dogs. Retrospectively it is quite possible that the substance was lipopolysaccharide, an endotoxin from gram-negative bacteria [4]. A milestone was the discovery of the connection between microorganisms and infectious diseases. Sepsis continued to be a syndrome with high morbidity and mortality up to the last century [3]. Pneumococcal pneumonia and septicemia were major killers among children and adults. A major breakthrough was the introduction of antibiotics in the first half of the last century [5]. Other breakthroughs came with introduction of vaccines and supportive therapy. Despite all these major milestones the mortality rate of sepsis has been relatively unchanged during the last 30-50 years.

Before 1991 there was no consensus in the definitions of different degrees of infection and sepsis. Terms like infection, bacteraemia, blood-borne infection, septicemia, sepsis, septic shock, septic syndrome and more were used. All these different terms and definitions probably reflected the immense heterogeneity in sepsis. Patients could have all kind of microbiological etiology to their sepsis, all kind of primary focus of infection, different degrees of severity and different degrees of co-morbidity. This lack of consensus made it very difficult to compare results from different studies and to plan new studies. A consensus committee was set to develop some criteria that could be used in sepsis. The committee agreed on the introduction of a set of criteria called the *Systemic Inflammatory Response Syndrome (SIRS)* criteria [6]. These criteria were: fever > 38º C; hypothermia < 36º C; tachycardia > 90 beats/minute; tachypnea > 20 breaths/minute (or PaCO2 < 4.3 kPa); leukocytosis > 12*10E9/l; leukopenia < 4*10E9/l (or > 10% immature bands). Sepsis was

defined as the combination of at least two SIRS criteria and the presence of infection (Figure 1). Severe sepsis was defined as sepsis with organ dysfunction, hypoperfusion or sepsis-induced hypotension. Septic shock was defined as severe sepsis with hypotension despite adequate fluid resuscitation.

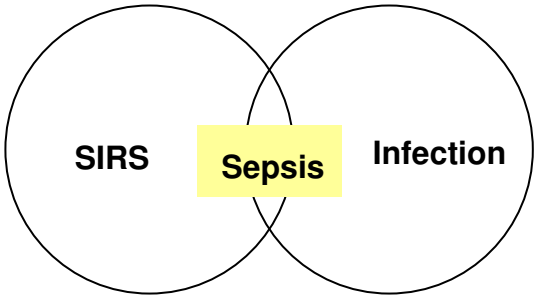

Figure 1

Sepsis is defined as a combination of SIRS and the presence of infection

Sepsis is a serious clinical condition with a high mortality [7]. The mortality rises up to 45% with increasing severity of sepsis [7]. The incidence of sepsis in the USA has increased from 82.7 per 100,000 population in 1979 to 240.4 per 100,000 population in 2000 [8]. The prevalence of severe sepsis among in-patients varies between 2 and 11% [9]. In a study examining the frequency of SIRS, 68% of the patients fulfilled the SIRS criteria at the time of admission [10]. Among these patients 26% developed sepsis, 18% severe sepsis and 4% septic shock [10]. Sepsis can be caused by different pathogens: bacteria, virus, fungus and parasites. Since mid-1980's gram-positive bacteria have been involved in the majority of sepsis cases [8]. The most frequent primary site of infection is the lung followed by the abdomen and the urinary tract [7].

Despite advances in therapy of infections in the form of introduction of potent antibiotics, dialysis therapy, intensive care and other measures the effect on the mortality rate of sepsis has been disappointing. Mortality has only been reduced in some subgroups of patients [10,11]. Prompt antibiotic therapy covering the pathogen involved, has been proved to reduce mortality [12]. During the last years several major studies have been completed with promising results regarding treatment of sepsis patients. In 2001 it was reported that *early-goal-directed therapy* could reduce mortality (absolute reduction) with 16% in patients admitted with sepsis [13]. The same year it was reported that intensive control of blood glucose on the intensive care unit could reduce mortality (absolute

reduction) with 3.4 % in surgical patients admitted to an intensive care unit [14]. The PROWESS study from 2001 examining activated protein C reported a mortality reduction (absolute reduction) of 6.1% in patients with sepsis and organ dysfunction [15]. In 2002 it was reported that low dose treatment with corticosteroids in patients with septic shock reduced mortality (absolute reduction) with 10% [16].

When the clinician wants to diagnose infection or sepsis he/she has to make a conclusion based upon several information sources. Most important are the medical history, objective findings, fever, clinical signs, laboratory investigations and radiological examination. An effective choice of antibiotic strategy depends on identification of the involved pathogen. Cultures with resistance examinations, microscopy, serological examinations and polymerase chain reaction are important techniques used in the clinical microbiology department. The biochemical laboratory tests used today are C-reactive protein, white blood cell total count and counting of leukocyte subtypes. These biochemical tests have been characterized by varying sensitivities and in general low specificities. These weaknesses have turned the focus on several new candidate diagnostic and prognostic markers in infection and sepsis [17-19]. These new markers have often been examined in prospective observational studies. Different abilities of these markers have been looked upon: the ability to identify infected patients, the ability to differentiate between SIRS without infection and sepsis and the ability to identify the presence of bacteraemia. Most diagnostic test studies focusing on sepsis have been done in intensive care departments. Because of the chosen intensive care unit settings in these studies most patients enrolled had either severe sepsis or septic shock. It is therefore difficult to use results from these studies on an internal medicine population, dominated by the milder end of the sepsis spectrum. If new markers of infection and sepsis are considered introduced on an internal medicine population, diagnostic test studies have to bee done in the same population.

During the last 10 to 15 years research has focused on the pro-inflammatory aspects of sepsis. SIRS criteria are based on the assumption that the systemic pro-inflammatory response is central in the pathogenesis of sepsis [6]. During the last years research has started focusing also on the anti-inflammatory aspects of sepsis [20]. This response is thought to follow the initial pro-inflammatory response with high levels of pro-inflammatory cytokines in the blood [21,22]. In this anti-inflammatory phase of sepsis high levels of anti-inflammatory cytokines (IL-4, IL-10), anergy in T-

cells and cellular apoptosis are observed [20]. This new knowledge points toward development of new methods to monitor the immune status in each patient with sepsis. If new therapeutic modalities (suppressing or stimulating the immune system) are supposed to work, we need to know the immune status of each patient. There have been many attempts to suppress the immune system in sepsis. These clinical studies have been disappointing although similar animal models have shown positive results [23]. Maybe these disappointing studies could be explained by the theory that patients in an anti-inflammatory phase have been exposed to immunosuppressive treatment. Maybe these patients could have benefited of an immune-stimulating treatment. There is no gold-standard for assessing the immune-status of a sepsis patient. Any new trial trying to develop a panel of molecular markers reflecting the immune status of the single patient has this problem of lacking gold-standard. Despite these challenges it is important continually to focus on possibilities to develop a method to evaluate the immunologic status of the patient with sepsis. The perspectives will be the possibility of tailoring a specific immune-stimulating or immunosuppressive therapy for each single sepsis patient.

V.2 Pro-inflammatory and anti-inflammatory aspects in sepsis

V.2.1 Pro-inflammation

Sepsis is considered to be a result of the host response to an infectious challenge (Figure 2) [24]. The SIRS criteria reflect the concept that sepsis was regarded primarily to be a pro-inflammatory response when the host is challenged by a pathogenic micro-organism [6]. A very complex response by the immune system, the coagulation system and several organs is seen in sepsis [24]. The innate immune system has a central role in the early phase of sepsis [25,26]. The cellular immune system and soluble elements of the immune system are both involved in the early innate immune response [27]. A complex network of pro-inflammatory cytokines, anti-inflammatory cytokines and other molecular markers are involved in the immunopathogenesis of sepsis [24]. Later on the adaptive immune system is also involved [25,28]. The homeostasis of the coagulation system is often disturbed with both thrombosis tendency and bleeding tendency occurring simultaneously [24]. Hypo-perfusion and hypotension results in organ dysfunction [24,29,30]. The infection can develop from a localized infection to sepsis and further on to severe sepsis and septic shock (Figure 3).

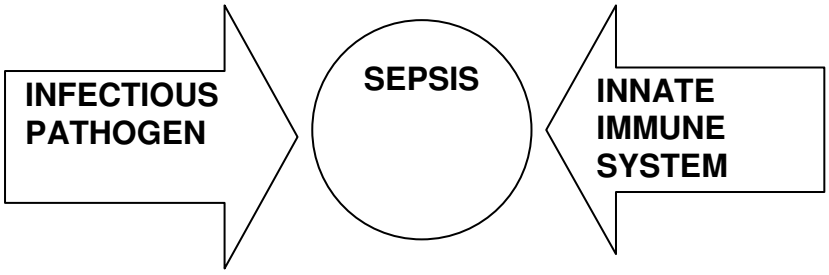

Figure 2

The development of sepsis depends on the interaction between the invading infectious pathogen and the innate immune system

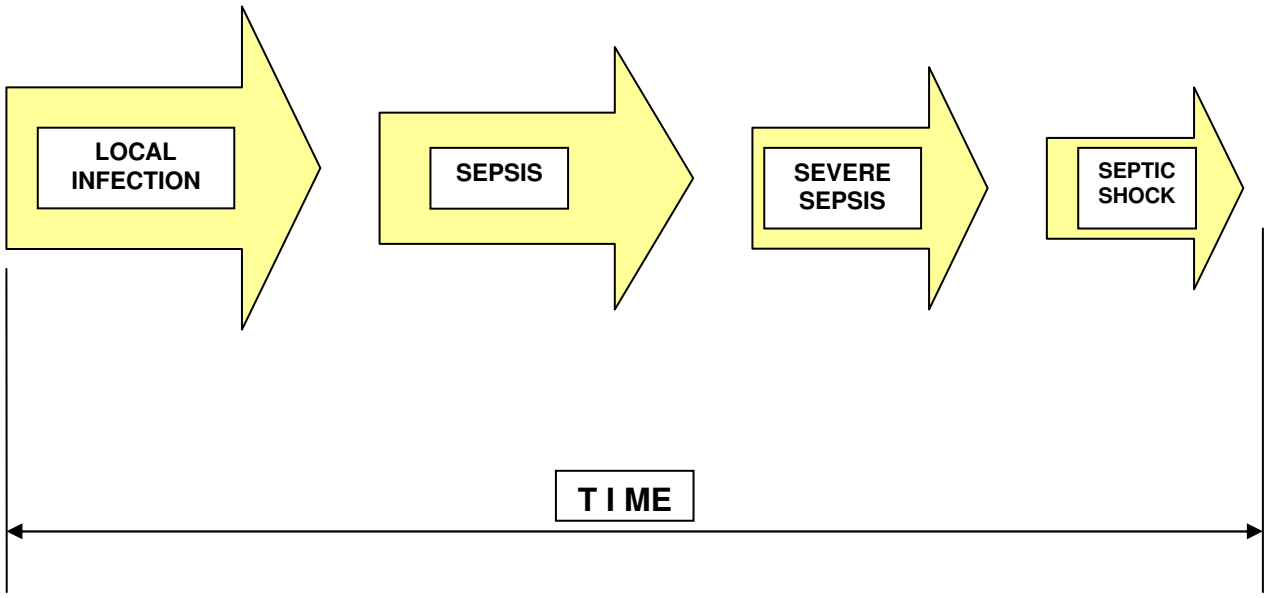

Figure 3

Infection and sepsis is a spectrum with increasing severity towards septic shock

The innate immune system is involved at an early stage when pathogenic micro-organisms are invading the host [26,31]. The innate immune system will respond to a bacterial or fungal challenge with a cellular defence [26]. Neutrophils, monocytes and macrophages have a central role [26]. These cells have membrane molecules called pattern recognition receptors (PRRs) (Figure 4). These PRRs have the ability to recognize molecular patterns on invading micro-organisms [26,32]. These

molecular patterns are called pathogen-associated molecular patterns (PAMPs) [33]. The interaction between PAMPs and PRRs will initiate an intracellular cascade of kinases which eventually will up-regulate nuclear-factor Kappa-B and finally up-regulate production of cytokines involved in the immune response [32]. An important group of PRRs are the Toll-like receptors (TLR) [32]. At least ten human TLRs have been described [32,34]. TLR-4 has a central role in binding lipopolysaccharide (LPS) delivered to the CD14 receptor by lipopolysaccharide-binding protein (LBP) [35]. TLR-4 is therefore an important PRR involved in the innate immune response in gram-negative infection [35]. TLR-2 is an example of a PRR involved in recognition of PAMPs from gram-positive bacteria [35]. Neutrophils, monocytes and macrophages are also effector cells involved in phagocytosis and killing of pathogens [28]. The adaptive immune system can be activated by macrophages and dendritic cells who present components of the pathogen to T-cells [36]. This will result in the possibility of developing protective immunity [36].

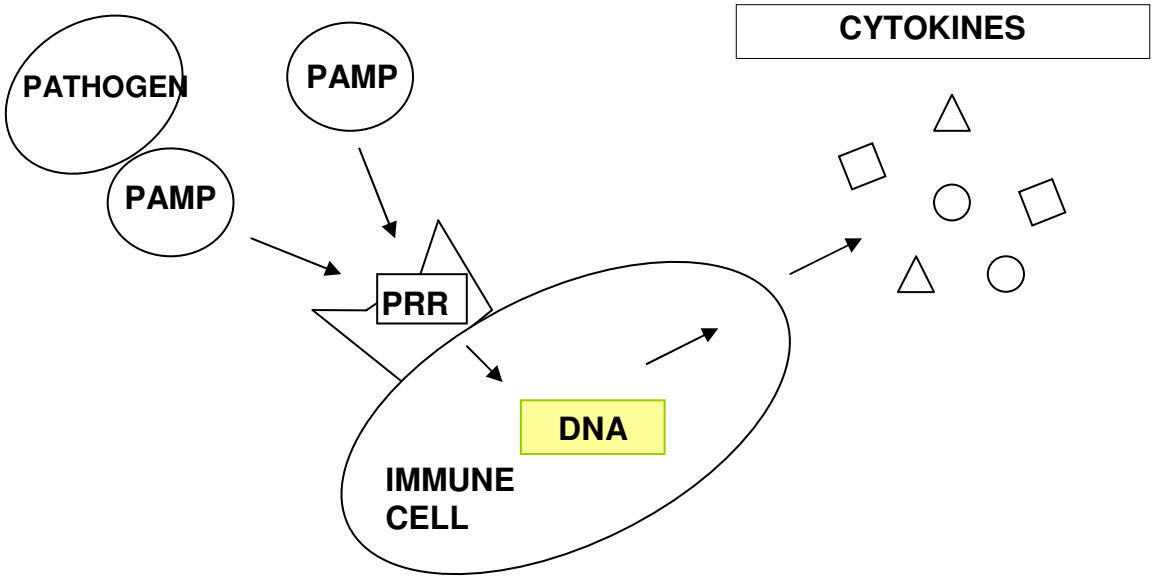

Figure 4

PAMPs from pathogens interact with PRRs on immune cells; this results in production of important cytokines

The activation of the innate immune system will result in production of many cytokines involved in the immune response [37-39]. Early in the developing sepsis the pro-inflammatory cytokines have

been given a key role [39]. Interleukin-1 (IL-1), TNF-alpha (TNF-a) and Interleukin-6 (IL-6) have been considered as important inflammatory cytokines in sepsis [39]. In recent years High-mobility group box 1 protein (HMGB1) has been in focus as an interesting pro-inflammatory cytokine [40]. As a late-onset pro-inflammatory cytokine HMGB1 has been given the possible role as the cytokine that maintains the pro-inflammation in sepsis [41].

Pro-inflammatory cytokines like IL-1 and IL-6 have the capability to increase the tissue factor expression [42]. This will increase production of prothrombin which will be converted to thrombin [29,30,43]. This will increase fibrinogen which will generate fibrin. This hyper-coagulability will induce thrombosis of small vessels and hypo-perfusion in many organs. The consumption of coagulation factors will also increase bleeding tendency in patients with sepsis. The anti-coagulation pathways will also be impaired by low levels of activated protein C and antithrombin [29,30,43].

Because of the central role of pro-inflammation in sepsis there have been many attempts to modify different aspects of the immune system in sepsis [23]. Animal models in the 1980s suggested that immunomodulating therapy could reduce the mortality in sepsis. Passive immunisation with anti-TNF-a was protective in animal models [44,45]. These studies were the background for initiating studies with immunomodulating therapy in patients [46]. Two prospective randomised trials studying the effect of high-dose corticosteroid therapy did not show any benefit on the mortality in sepsis patients [47,48]. One large trial with anti-TNF-a therapy did not show any benefit [49]. Two recently conducted trials involving therapy with low-dosis corticosteroids and with activated protein C have shown a positive effect on the mortality rate [15,16]. Overall the human sepsis studies focusing on immunomodulation of the pro-inflammation in sepsis have been quit disappointing. Different explanations on these failures have been proposed. There has been critic of the animal models based on LPS stimulation without a bacterial challenge. These models probably induced unrealistic high levels of TNF-a, not reflecting clinical reality [50]. Animal models using cecal ligation and puncture showed much lower TNF-a levels after the insult [51]. In these models anti-TNF-a therapy did not improve survival in the animals [51]. The heterogeneity among patients included in trials focusing on immunomodulatory therapies has been considered to be one reason for the disappointing results [50]. The expectation that all patients were in a pro-inflammatory state has also been questioned [50]. If some of the patients were in a anti-inflammatory state the

experimental therapies might have done more harm than good [50]. In opposition to the animal models which are conducted under very controlled circumstances, human patients have often been ill for many hours, maybe several days, before enrolment in studies. An intervention with immunomodulatory therapy may be far too late compared to the moment were the innate immune system was challenged by the pathogen.

V.2.2 Anti-inflammation

The definition of sepsis as the host response to an infectious insult and the introduction of the SIRS criteria in 1991, emphasised the pro-inflammatory state in sepsis. This simplistic model combined with the disappointing results in immunomodulatory therapy trials focusing on dampening of the inflammatory response, pointed towards a new hypothesis. Roger C. Bone introduced a new concept in 1996 called *compensatory anti-inflammatory response syndrome (CARS)* [52]. This hypothesis suggested that both SIRS and CARS could be present in different phases of sepsis or in parallel [52]. If CARS was severe enough this could be manifested with for i.e. increased susceptibility to infection [52]. The presence of CARS could maybe explain the disappointing results from immunosuppressive therapy in clinical sepsis trials [23,50]. Studies have shown that low levels of HLA-DR expression on monocytes from sepsis patients, that could reflect a hypo-immune state, were associated to a higher mortality [53,54]. Monocytes from sepsis patients, with poor ex-vivo ability to synthesise pro-inflammatory cytokines after LPS stimulation, were a negative prognostic factor [53]. These studies suggest that there are conditions where sepsis patients can have some kind of immune-paresis. Studies have shown higher survival rate among sepsis patients with immune-paresis treated with interferon-gamma [53]. Studies have shown increased levels of the anti-inflammatory cytokine interleukin-10 (IL-10) in sepsis and high levels predicted mortality [55,56]. Studies have shown anergy of T-cells in peritonitis [57]. T-cells from sepsis patients had reduced capability of mounting at Th1 response (TNF-a, interferon-gamma, interleukin-2) [57]. Apoptosis is another phenomenon observed in sepsis [58-60]. Large numbers of lymphocytes are eradicated by apoptosis in sepsis [60]. All the abovementioned mechanisms tend to move the sepsis patient in direction to an immune-suppressed state (Figure 5).

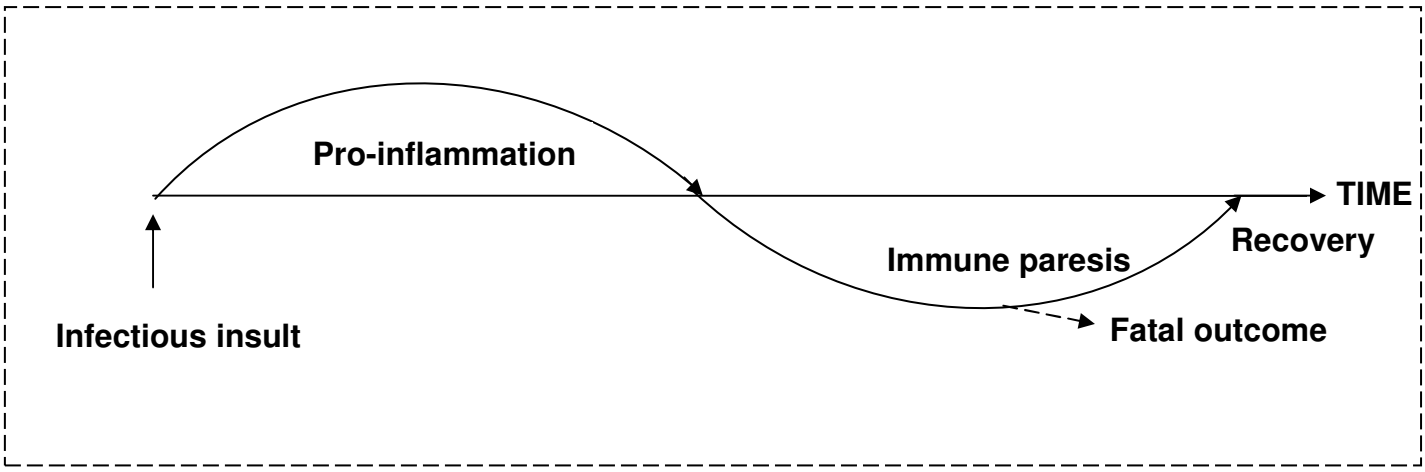

Figure 5

Simple hypothetical model illustrating pro-inflammation and immune paresis as consecutive phases in the patient with sepsis

V.2.3 Both SIRS and CARS in parallel

In the hypothesis by RC Bone regarding CARS it was assumed that homeostasis could be maintained by a balance between SIRS and CARS [52]. In a complex disease like sepsis it is possible that both pro-and anti-inflammation can be present at the same time. A pro-inflammatory state can be present in the infectious focus while there is a systemic anti-inflammatory state [61]. This has been shown in patients with acute appendicitis where there were elevated levels of pro-inflammatory cytokines in the peritoneal fluid while at the same time there were elevated levels of anti-inflammatory cytokines in plasma [61]. IL-10 levels in plasma were related to the severity of appendicitis [61].

It is quit clear that a simplistic model only focusing on the hyper-inflammation in sepsis does not reflect clinical reality. The knowledge on the anti-inflammatory aspects in sepsis acquired during the last ten years demonstrates a need for more complex models for understanding the immunopathogenesis in sepsis. Maybe patients could be benefited of systems to monitor the immune status of each single patient with sepsis. One method could be a panel of biomarkers reflecting the immune status. This could be parallelized to the usage of coronary markers in diagnosis and treatment of acute coronary syndrome and the usage of tumour markers in cancer therapy. An effective *immune status monitoring system* would make it possible to stratify patients in sepsis trials and maybe enable therapy to be tailored to each single sepsis patient. Studies focusing

on developing immune status monitoring systems have the lack that there is no gold-standard for immune status.

V.3 Diagnostic and prognostic markers in infection and sepsis

The clinician has several biochemical, microbiological and radiological examination modalities to supplement the medical history, objective examination and the classical inflammatory signs of *rubor*, *calor*, *dolor* and *tumour* (Figure 6). C-reactive protein (CRP), white blood cell (WBC) count and subtype counting of WBC are used in the clinic to diagnose inflammation and infection [62,63]. Other acute phase proteins (APPs) like serum amyloid A, ferritin and several others can also increase with inflammation [64]. All these markers are characterised by varying sensitivities and low specificities.

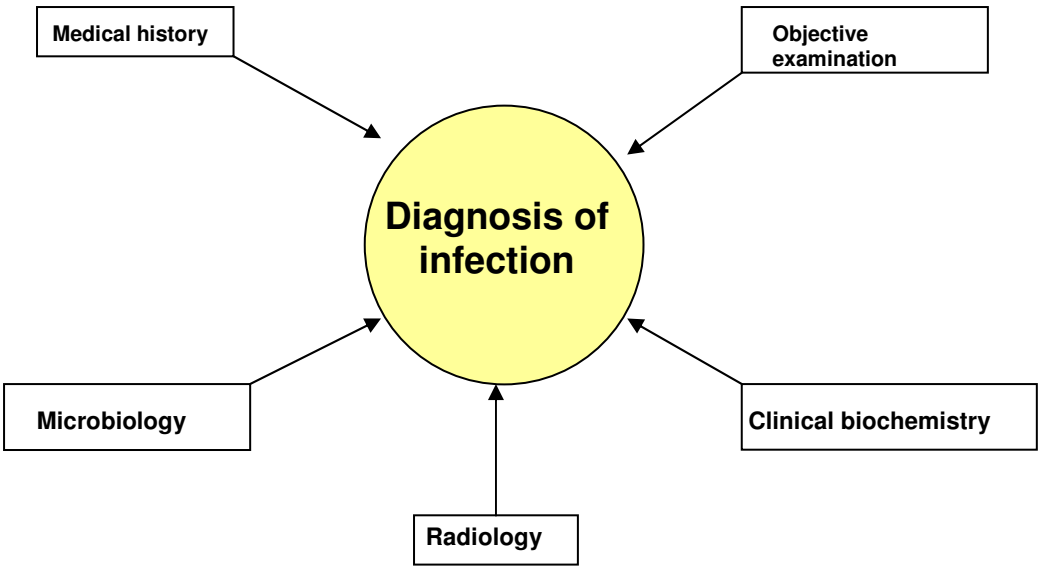

Figure 6
Data sources used in diagnosing infection and sepsis

Several new candidates for diagnostic and prognostic markers in infection and sepsis have been proposed during the last 10-15 years: Procalcitonin (PCT), Lipopolysaccharide-binding protein

(LBP), IL-6, sCD163, HMGB1, human adrenomedullin (AM) and other molecular markers including different cytokines [18,41,65-67]. PCT has already been implemented especially within critical care in some European countries.

Several qualities should be required from a diagnostic and/or prognostic marker in infection and sepsis. The diagnostic test abilities should have been thoroughly tested on the same population that the test is intended to be used on. An acceptably high sensitivity and specificity to identify infected and non-infected patients should be required. The diagnostic abilities should at least be as good as the best test used in clinical routine today. Other non-diagnostic abilities should also be examined: prognostic abilities, antibiotic saving abilities, abilities to reduce the length of hospital admission, morbidity reducing abilities, abilities to reduce the amount of invasive procedures and operations, economical cost-benefit. The assay characteristics should be known in detail. The assay should be possible to perform on a routine basis in a hospital laboratory. The assay time should be short so the clinician has a fast laboratory response to act on at the clinical setting.

V.4 Markers

V.4.1 Procalcitonin (PCT)

Assicot and colleagues described in 1993 an association between bacterial systemic infections and elevated levels of PCT [68]. PCT is a 116 amino acids (aa) prohormone in the synthesis of calcitonin. PCT has a molecular weight of approx. 14 kDa [69]. Different parenchymal tissues from all parts of the body have been shown to produce PCT when the body is challenged by microbial infection [65]. The liver, lungs, kidneys, muscles and adipose tissue are principal sources of circulating PCT [65,70-72]. Several studies have shown that PCT has a role in the immunopathogenesis of sepsis. Animal models with hamsters have shown that PCT levels correlate to the severity and mortality in sepsis models [73]. Another study with hamsters with bacterial peritonitis showed elevated levels of PCT in infected hamsters [74]. Administration of exogenous PCT to septic hamsters increased mortality and administration of antiserum against PCT protected against fatal outcome [74]. This study concluded that PCT was a marker of severity, had a role in the inflammatory process, and affected mortality in septic animals [74]. Another study with septic hamsters pointed towards a role for PCT as a secondary inflammatory mediator that could increase but not initiate the septic inflammatory process [75]. Data from another study suggested that PCT

levels were not dependent on the systemic presence of TNF-a in sepsis among baboons [76]. Two studies focusing on an animal model with septic pigs showed that both late and early immunoneutralization of PCT attenuated the adverse physiologic response in these sick animals [77,78]. The authors suggested that these observations maybe could generate treatment options in human sepsis [77,78]. Many studies have looked upon PCT levels in different infectious and inflammatory conditions. Elevated levels of PCT have been observed in toxic shock syndrome, bacterial sepsis, postoperative infectious complications, infection and rejection after solid organ transplantation, bacterial meningitis, cholangitis, pancreatitis with infected necrosis, acute malaria and candidemia [79].

Most studies focusing on PCT levels and diagnostic test abilities in sepsis have been conducted on intensive care unit populations [80-85]. These studies were thus dominated by patients with severe sepsis and septic shock with high SOFA and APACHE scores. Few studies have looked upon levels and diagnostic test abilities in the milder end of the sepsis spectrum (Table 1) [86-92]. These patients are often treated on internal medicine departments and not on ICU's. Levels of PCT in non-critically ill infection/sepsis patient populations are reported in Table 2 [86-88,93-96].

Three systematic reviews and meta-analyses have been conducted regarding PCT as a diagnostic test, as a marker of bacterial infection and regarding the diagnostic accuracy for sepsis diagnosis [97-99]. Two of these studies concluded that PCT was superior to CRP as a marker of bacterial infection and as a diagnostic test for sepsis in critically ill patients [97,98]. One of these studies did not support the widespread use of PCT in the critical care setting [99]. Only few studies have focused upon the diagnostic test abilities of PCT in diagnosing infection and sepsis in patients in the milder end of the sepsis spectrum. These studies found sensitivities between 24% and 77% and specificities between 70% and 94% (Table 1) [86-90; 92]. Several studies have examined PCT's abilities in diagnosing the presence of bacteremia (Table 3) [100-107].

Table 1

Diagnostic test abilities of PCT in identifying non-ICU patients with infection

Infected (n)	Non-infected (n)	Infection	Setting	Cut-off	Sensitivity (%)	Specificity (%)	AUC	Ref.
33	27	CAP	ID dept.	0.5 ng/ml	61.0	92.0		86
30	27	pyelonephritis	ID dept.	0.5 ng/ml	44.0	92.0		86
68	127	infected	ED	0.1 ng/ml	74.0	74.0	0.79	87
58	49	infected	ED	0.6 ng/ml	70.7	63.3	0.69	88
39	20	infected	ID dept.		54.0	70.0		89
50	168	infected	[a]	0.5 ng/ml	24.0	94.0		90
96	34	infected	ID dept.				0.72	91
167	77	bacterial/parasitic	ED	0,2 ng/ml	77.0	59.0	0.77	92

AUC, Area under the curve; CAP, Community-aquired pneumonia; ID dept., Dept. of Infectious Diseases; ED, Emergency department. [a]Dept. of Geriatrics.

Table 2

Levels of procalcitonin in non-ICU infected patient populations

Patients (n)	Controls (n)	Setting	Infection	PCT in patients (median)	PCT in controls (median)	Ref.
96	0	ID dept.	CAP	2.27 µg/l		93
33	27	ID dept.	CAP	0.88 ng/ml	0.21 ng/ml	86
30	27	ID dept.	pyelonephritis	0.46 ng/ml	0.21 ng/ml	86
68	127	ED	CA infections	5.3 ng/ml [a]	0.09 ng/ml [a]	87
58	49	ED	CA infections	0.67 ng/ml	0.5 ng/ml	88
185	0	ED	CAP	0.1 µg/l		94
20	60	multicenter	systemic infection	0.66 ng/ml	0 ng/ml	95
70	60	multicenter	localized infection	0.94 ng/ml	0 ng/ml	95
26	60	multicenter	nonbacterial infection	0.16 ng/ml	0 ng/ml	95
69	60	multicenter	suspected infection	0.38 ng/ml	0 ng/ml	95
116	24	ED	CAP	0.74 ng/ml (S. pneumoniae)	0.35 ng/ml	96

PCT, procalcitonin; Ref., Reference; CAP, Community-aquired pneumonia; ID dept., Dept. of Infectious Diseases; community-aquired pneumonia; ED, Emergency department; CA infections, community-aquired infections. [a]Mean value.

Table 3

Diagnostic test abilities of PCT in identifying patients with bacteremia

Bacteremic patients (n)	non-bacteremic patients (n)	Setting	Cut-off level	Sensitivity (%)	Specificity (%)	AUC	Ref.
13	16	internal medicine	1.9 µg/l	80.0	91.0	0.84	100
50	150	ID dept.	0.2 ng/ml	92.0	43.0		101
22	143	ID dept.	0.4 ng/ml	95.2	57.4	0.83	102
12	21	ICU	3.03 ng/ml	83.0	48.0	0.80	103
13	89	ED	0.4 ng/ml	92.0	70.0	0.85	104
21	73	haematology[a]	0.5 ng/ml	58.0	83.0	0.71	105
18	35	haematology[a]	0.62 ng/ml	72.0	77.0	0.75	106
14	94	ED	0.2 ng/ml	93.0	38.0		107

PCT, procalcitonin; AUC, area under the curve; Ref, reference; ID dept, Dept. of Infectious Diseases; ICU, Intensive Care Unit; ED, Emergency department. [a]Febrile neutropenia.

V.4.2 Lipopolysaccharide binding protein (LBP)

LBP was isolated from rabbit acute phase serum in 1986 [108]. LBP is a 452 aa polypeptide with a molecular weight of 50 kDa [108]. It has a well established role in the early innate immune response when the host is challenged by gram-negative pathogens [109,110]. LBP binds to the endotoxin lipopolysaccharide (LPS) and brings LPS to the CD14 receptors on the monocyte-macrophage cell lineage. CD14 receptors then interact with Toll-like receptor-4, initiating cytokine production [109,110]. LBP‘s role is not restricted to gram-negative infections. LBP is a general recognition molecule involved both in gram-negative and gram-positive infections [108]. Several bacterial surface components from gram-positive bacteria are recognized by LBP [108]. The lipoteichoic acid from pneumococci and *Staphylococcus aureus* activates a cellular response through Toll-like receptor-2. This response can be enhanced by LBP and CD14 [111]. LBP recognizes major inflammatory components of pneumococci, the pneumococcal cell wall as well as intact heat killed pneumococci [112]. LBP is synthesized in the liver, epithelial cells, lungs, intestine, gingival tissue, muscle cells and renal cells [108]. IL-1 or IL-1 and IL-6 in synergy enhance synthesis of LBP in the liver [108]. TNF-a and dexamethason can also enhance synthesis of LBP [108]. High levels of LBP in the serum of infected hosts can inhibit the LPS-induced innate immune system response [108,113]. This paradox can maybe be explained by the ability of LBP to transfer LPS to serum lipopolyproteins [108]. This could neutralize the bioactivity of LPS in the infected host’s serum [108].

Few studies have focused on levels of LBP in infected adult patients [114-119]. The levels of LBP reported in these studies are shown in Table 4.

Only two studies have reported on the diagnostic test abilities of LBP in adult patients with infections [117,118]. Oude Nijhuis and colleagues reported a sensitivity of 100% and specificity of 92% when they evaluated LBP's ability to identify neutropenic cancer patients (children and adults) with gram-negative bacteremia . They used a high cut-off level (46.3 μg/ml) for LBP [117]. Prucha and colleagues reported a sensitivity of 50% and specificity of 74.2% in discriminating between non-infectious SIRS and sepsis, in a cohort of patients requiring intensive care [118].

Table 4

Levels of Lipopolysaccharide-binding protein in infected patient populations

Patients (n)	Controls (n)	Setting	Infection	LBP in patients (median)	LBP in controls (median)	Ref.
10	10	ICU	gram-negative	range: 735-1252 nmol/l	85 nmol/l[a]	114
253	33	ID dept.	sepsis	31.2 μg/ml	4.1 μg/ml[a]	115
37	18	ICU	sepsis	46.4 μg/ml[a]	5.7 μg/ml[a]	116
40	23	ICU	SIRS	30.6 μg/ml	no data in article	118
19	23	ICU	sepsis	37.1 μg/ml	no data in article	118
9	23	ICU	septic shock	59.7 μg/ml	no data in article	118
4	0	oncology	gram-negative bacteremia	54.2 μg/ml		117
14	0	oncology	gram-positive bacteremia	21.1 μg/ml		117
48	0	oncology	FUO	21.2 μg/ml		117
36	49	ICU	gram-negative bacteremia	228 μg/ml	16.2 μg/ml	119
28	49	ICU	gram-positive bacteremia	203 μg/ml	16.2 μg/ml	119

LBP, Lipopolysaccharide-binding protein; ICU, Intensive care unit; ID dept., Dept. of Infectious Diseases; FUO, Fever of unknown origin. [a]mean value.

V.4.3 Soluble haemoglobin scavenger receptor (sCD163)

Scavenger receptors belong to the group of pattern recognition receptors [120]. Scavenger receptors are characterized by their broad range of ligand binding [120]. On sub-group of scavenger receptors are the scavenger receptor cystein-rich (SRCR) family [120]. Some of these SRCR family molecules have a role in the innate host defence by acting as pattern recognition molecules [120]. CD163 (previously called RM3/1 antigen or M130) is a membrane bound SRCR exclusively expressed in the monocyte-macrophage cell lineage [121]. CD163 is a glycoprotein with a molecular weight of 130 kDa [122]. The expression of CD163 has been shown to be down-regulated by pro-inflammatory mediators like LPS, interferon-gamma and TNF-a [123-125]. CD163 has been shown to be up-regulated by anti-inflammatory mediators like glucocorticoids, IL-6 and IL-10 [123-125]. CD163 is involved in endocytosis of haptoglobin-haemoglobin complexes [122]. Heme and heme proteins are known to have pro-inflammatory effects [126] (Figure 7A & 7B). When monocytes are stimulated to an "alternative" phenotype with the anti-inflammatory cytokines Interleukin-4 and Interleukin-13, high expressions of CD163 are observed in these "alternatively" activated monocytes [120,127,128]. Soluble haemoglobin scavenger receptor (sCD163) is slightly smaller than the membrane-bound CD163, suggesting the shedding mechanism to be a proteolytic cleavage-dependent process [129]. The role of sCD163 has not been elucidated yet. Elevated levels of sCD163 have been observed in several clinical conditions like pneumonia/sepsis in haematological patients, mononucleosis, leishmaniasis, myelomonocytic leukaemia, reactive haemophagocytic syndrome, Gaucher's disease and liver failure [129-132].

Only three previous studies have reported on levels of sCD163 in adult sepsis patients [129,131,133]. In the first mentioned study, mean levels of sCD163 of 8 mg/l were observed among patients with pneumonia/sepsis [129]. In a study with different infections including severe sepsis, mean levels of sCD163 of 9.1 mg/l were observed among patients with severe sepsis [131]. In another study mean levels of sCD163 of 7 mg/l were observed among patients with pneumococcal bacteremia [133]. There have been no publications regarding the diagnostic test abilities of sCD163 in diagnosing severe infections/sepsis.

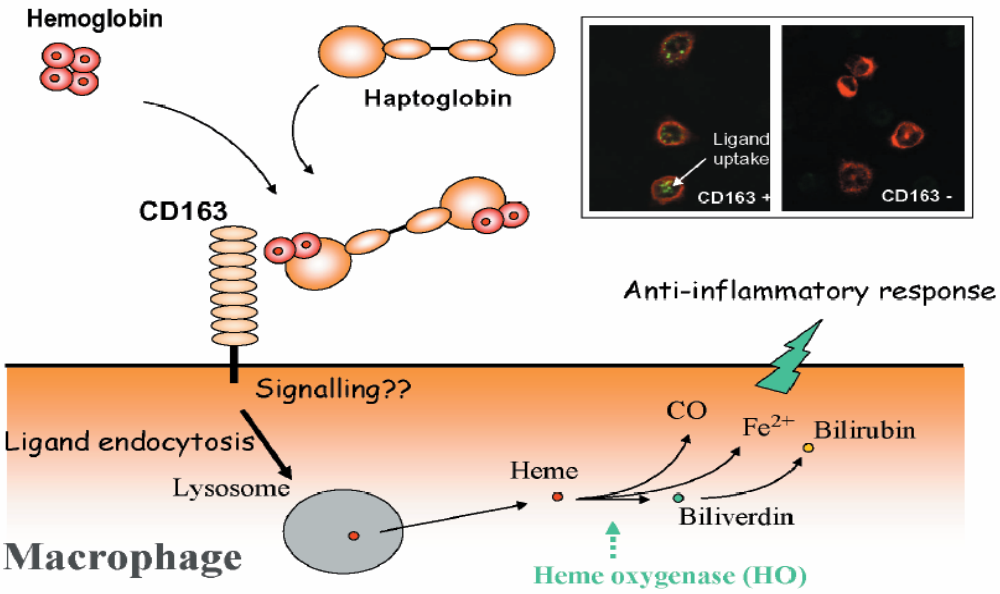

Figure 2. Binding of hemoglobin to haptoglobin and uptake of the complex by CD163 in macrophages. Hemoglobin tetramers disrupt into αβ dimers, which bind to the β-chain of haptoglobin. Endocytosis of the receptor ligand complex leads to degradation of the protein part of the ligand whereas the receptor is suggested to recycle. The heme molecule of hemoglobin is converted by the heme-oxygenase to the anti-inflammatory metabolites CO, biliverdin and Fe^{2+}. Interleukin-6 (IL-6) is a known regulator of the pathway by stimulating the synthesis/expression of haptoglobin, CD163 and heme oxygenase-1 (HO-1). Induction of an intracellular signaling cascade leading to increased HO-1 activity and secretion of anti-inflammatory cytokines such as IL-10 may reinforce an anti-inflammatory response (26). The inset (upper right) shows uptake of Alexa488 (green fluorescence)-labeled haptoglobin-hemoglobin complexes in CD163-transfected cells, Chinese hamster ovary cells (CD163+) and control cells (CD163−) transfected with an irrelevant receptor protein (cubilin). The receptor proteins (CD163 in left panel, cubilin in right panel) are immunostained with a specific monoclonal antibody and an Alexa594-labeled (red fluorescence) secondary antibody. Only the cells transfected with CD163 take up the ligand (haptoglobin-hemoglobin complexes) seen as a green vesicular staining.

Figure 7A: CD163 has a central role in the metabolism of haemoglobin

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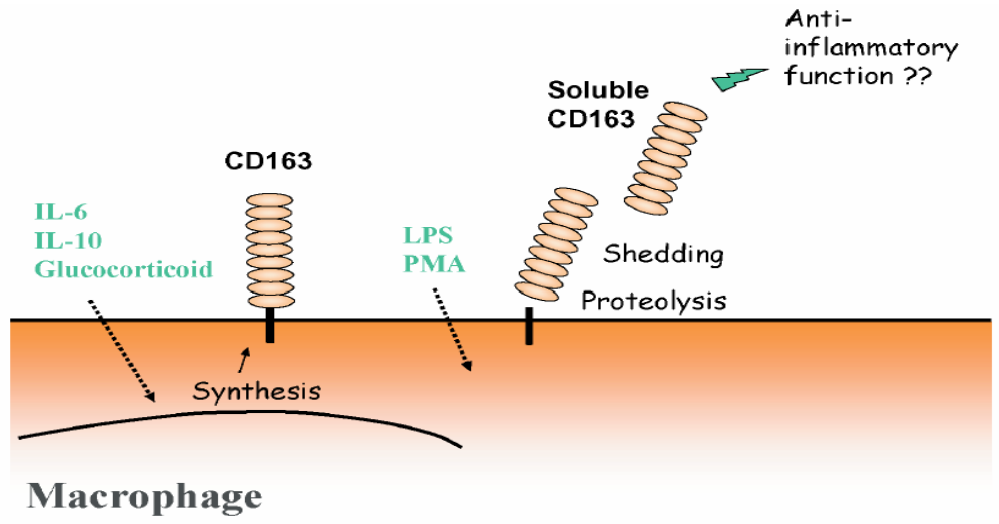

Figure 3. Regulation of CD163 synthesis and shedding. Synthesis and expression of CD163 are induced by glucocorticoid, IL-6, and IL-10. A protease-dependent cleavage of the extracellular scavenger domains from the cell surface can be induced by phorbol esters. The soluble form of CD163 might be involved in anti-inflammatory functions, e.g., inhibition of lymphocyte activation and proliferation. The punctuated arrows indicate the stimulatory effect of various substances on CD163 synthesis and shedding, respectively.

Figure 7B: Regulation of CD163 synthesis and shedding

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V.4.4 High mobility group box-1 protein (HMGB1)

HMGB1 was described more than 30 yrs ago as nuclear chromosomal protein [40]. HMGB1 is a 215 aa protein that has been shown to be highly conserved among different species [40]. During the last years there has been an increased interest on other roles for this protein. In an attempt to identify "late-onset" cytokines/mediators in sepsis, HMGB1 was identified as a candidate for this role [134]. The background for these studies were several earlier disappointing studies, in which blockage of early pro-inflammatory pathways had been tried with no success in patient cohorts with sepsis [23]. An attempt was made to identify mediators that were up-regulated later on in the inflammatory process that followed the interaction between a pathogenic micro-organism and the host's innate immune system. In an in-vitro system with cultured macrophages stimulated with endotoxin/TFN-a/IL-1; HMGB1 was identified as a possible "late-onset" cytokine [134] (Figure 8). The same group observed increasing levels of HMGB1 8-32 h after that mice were exposed to endotoxin [134]. Administration of antibodies to HMGB1 to endoxin exposed mice reduced the mortality and administration of HMGB1 to the same mice promoted a lethal course in this sepsis animal model [134]. HMGB1 has been shown to have many organ specific biological functions such as induction of fever, anorexia, weight loss and cytokine production in the brain; induction of acute lung injury and production of pro-inflammatory cytokines in the lungs; promotion of translocation in the gut; induction of arthritis and joint inflammation; affection of the heart rhythm; and having bactericidal effects [135] (Figure 9).

Only 5 previous studies have reported on HMGB1 levels in severe infections/sepsis [134,136-139]. In a study by Wang and colleagues HMGB1 was measured with an immunoblotting method in 8 healthy controls and in 25 patients with sepsis [134]. The highest levels of HMGB1 (median 84 ng/ml) were observed among sepsis patients with fatal outcome [134]. Patients surviving sepsis had HMGB1 median of 25 ng/ml and healthy controls had undetectable levels of HMGB1 [134]. In a prospective observational study on ICU patients with severe sepsis and septic shock, levels of HMGB1 and several cytokines were measured with two different immunoblotting methods over several days [136]. HMGB1 levels remained elevated in the majority of these critically ill sepsis patients in up to a week after inclusion [136]. Levels of HMGB1 were elevated for a longer time compared to other measured cytokines (IL-6, Interleukin-8, IL-10 and TNF-a) [136]. In a large prospective study measuring HMGB1 levels with an immunoblotting method in patients with community-acquired pneumonia median levels of 190 ng/ml were found in infected patients [138].

In a study measuring HMGB1 levels with an ELISA the following HMGB1 levels were observed in different subgroups of patients: undetectable levels in healthy controls, mean 4.54 ng/ml in infected patients, mean 2.15 ng/ml in malignant disease, mean 6.47 ng/ml in trauma patients, mean 14.05 ng/ml in patients with disseminated intravascular coagulation [137]. In another study measuring HMGB1 levels in patients with septic shock the observed median HMGB1 concentration was 4.4 ng/ml [139].

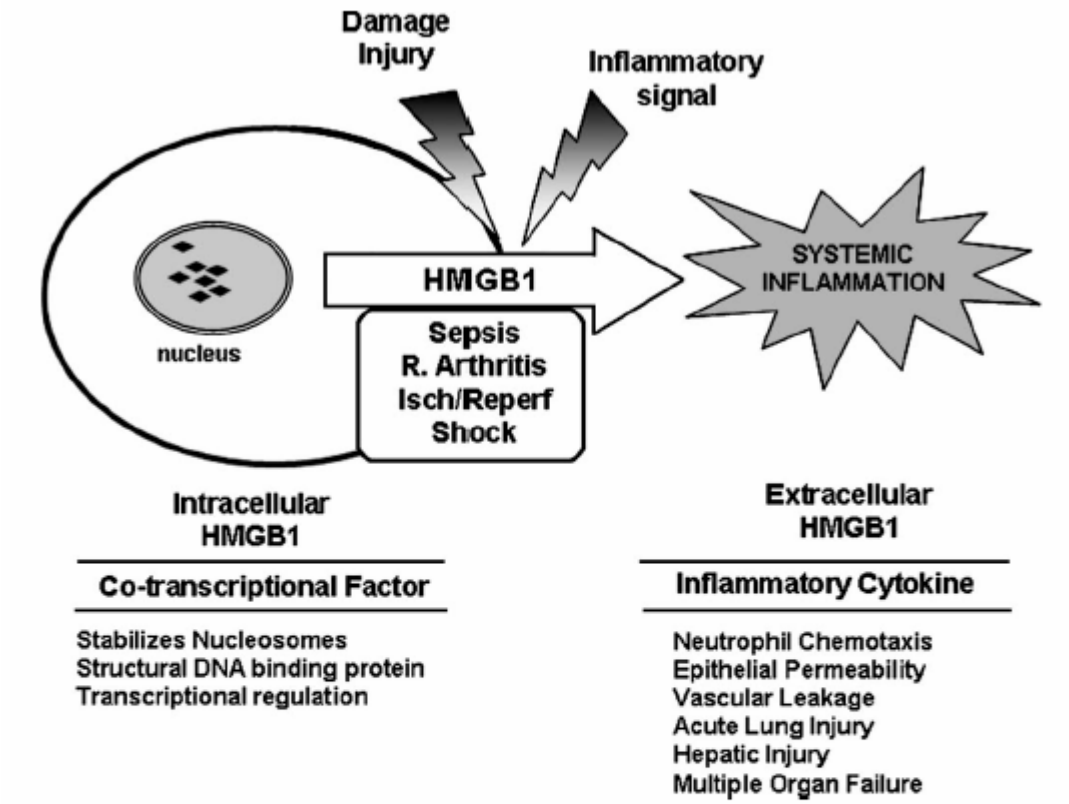

FIG. 1. **HMGB-1 is an inflammatory cytokine.** High-mobility group box (HMGB)-1 was originally described as a nuclear DNA-binding protein that functions as a structural cofactor critical for proper transcriptional regulation and gene expression. Recent studies indicate that damaged, necrotic, or activated immune cells liberate HMGB-1 into the extracellular milieu, where it functions as a proinflammatory cytokine and contributes to the pathologic progression of diverse infectious and inflammatory disorders. HMGB-1 represents a novel family of inflammatory cytokines composed of intracellular proteins that, when present in the extracellular milieu, are recognized by the innate immune system as a signal of tissue damage or immune activation.

Figure 8

Dual role of HMGB1 as a nuclear protein and as an inflammatory cytokine

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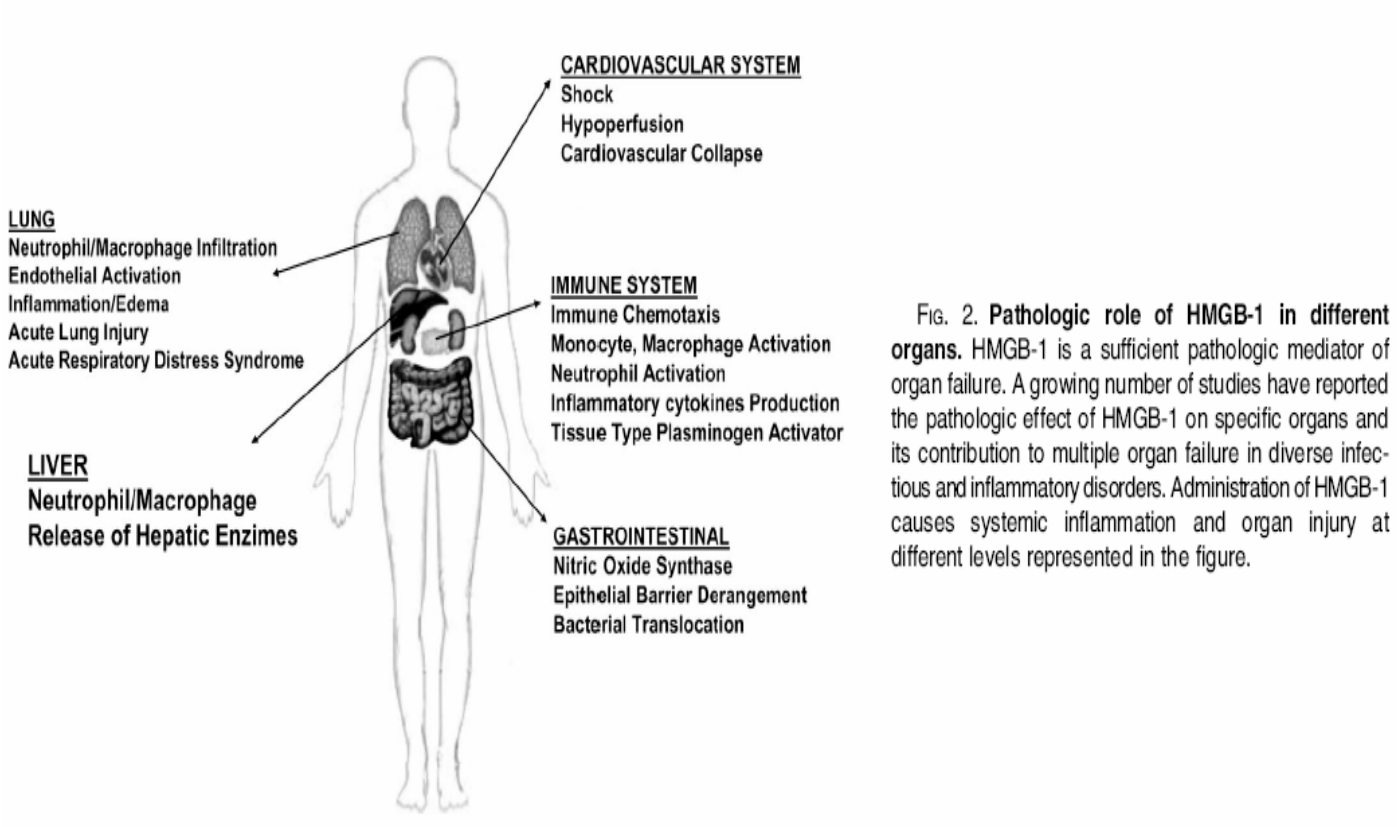

FIG. 2. **Pathologic role of HMGB-1 in different organs.** HMGB-1 is a sufficient pathologic mediator of organ failure. A growing number of studies have reported the pathologic effect of HMGB-1 on specific organs and its contribution to multiple organ failure in diverse infectious and inflammatory disorders. Administration of HMGB-1 causes systemic inflammation and organ injury at different levels represented in the figure.

Figure 9

HMGB1 role in different organs

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V.4.5 C-reactive protein (CRP), Interleukin-6 (IL-6), white blood cell (WBC) count, neutrophils, soluble triggering receptor expressed on myeloid cells-1 (sTREM1) and adrenomedullin (AM)

CRP is a pentameric protein with a molecular weight of approx. 115 kDa [140]. It was identified in serum from patients with pneumococcal infections [140,141]. CRP is a human acute phase protein with important roles as a pattern recognition molecule and with an important role in the innate immune system [64,141]. CRP has the capability to interact with a variety of ligands: phosphocholine in several bacterial species, phosphocholine in eukaryotic membranes, phosphoethanolamine, chromatin, histones, fibronectin, small nuclear ribonucleoproteins, laminin and polycations [141]. CRP can activate the classical complement pathway, stimulate phagocytosis and bind to immunoglobulin receptors [141]. Several studies have examined the diagnostic test abilities of CRP in sepsis patients admitted to emergency departments and departments of internal medicine (Table 5) [86,88,90-92].

Table 5

Diagnostic test abilities of CRP in identifying non-ICU patients with infection

Infected (n)	Non-infected (n)	Infection	Setting	Cut-off	Sensitivity (%)	Specificity (%)	AUC	Ref.
33	27	CAP	ID dept.	50 mg/l	91.0	33.0		86
30	27	pyelonephritis	ID dept.	50 mg/l	94.0	33.0		86
58	49	infected	ED	60 mg/l	67.2	93.6	0.88	88
50	168	infected	[a]	3 mg/l	92.0	36.0		90
96	34	infected	ID dept.				0.81	91
167	77	bacterial/parasitic	ED	40 mg/l	76.0	62.0		92

AUC, Area under the curve; CAP, Community-aquired pneumonia; ID dept., Dept. of Infectious Diseases; ED, Emergency department. [a]Dept. of Geriatrics.

IL-6 is a pleiotropic cytokine identified in 1980 [142]. It is produced both in non-lymphoid tissue and in lymphoid tissue [142]. IL-6 has several biological functions: regulates immune reactivity, stimulates production of acute phase proteins like CRP and LBP, involved in oncogenesis and in hematopoiesis [142]. IL-6 levels are elevated in many different clinical conditions (injury, trauma, stress, infectious insults, brain death) [142]. IL-6 production is stimulated by early pro-inflammatory cytokines like TNF-alpha and Interleukin-1. Compared to these “early-onset” pro-inflammatory cytokines with very short half-lives IL-6 levels are elevated for much longer periods in severe infections/sepsis [142]. A correlation between IL-6 levels and the severity/mortality of

sepsis has been observed in several studies [143-145]. Sensitivities between 65 % and 86 % and specificities between 54 % and 79 % have been found in diagnosing sepsis [83,85,95].

WBC count and leukocyte differential count are laboratory parameters that have been used for many years in both out-patients and in-patients [146]. Very few studies have put attention to these markers in relation to infections and sepsis during the last years. One recent study showed that neutrophils had an AUC of 0.74 in diagnosing bacterial infection in non-critically ill sepsis patients [91].

Triggering receptor expressed on myeloid cells (TREM) is a family of surface receptors involved in different biological functions: inflammation, bone homeostasis, development of the nervous system and coagulation [147]. Triggering receptor expressed on myeloid cells-1 (TREM1) is a receptor involved in the activation of monocytes and in the inflammatory responses [148]. The innate inflammatory response involving activation through TLR-2 and TLR-4 is enhanced by the engagement of TREM1 [148]. The expression of TREM1 has been shown to be enhanced in sepsis [148]. Infection induces release of a soluble form of soluble triggering receptor expressed on myeloid cells-1 (sTREM1). sTREM1 has been shown to be elevated in bronchoalveolar fluid from patients with pneumonia requiring mechanical ventilation and in plasma of sepsis patients at the intensive care unit [149,150]. In the pneumonia study sTREM1's Area under the curve (AUC) in diagnosing infection was 0.93 [149]. In the sepsis study sTREM1's AUC in diagnosing infection was 0.97 [150]. Studies examining less ill patients with pneumonia and sepsis have shown poorer diagnostic test abilities in diagnosing infection with sTREM1 [91,151].

AM is a peptide identified in 1993 from human pheocromocytoma [152]. AM seems to be involved in several important biological functions like blood pressure regulation, protection against organ dysfunction/damage in sepsis and hypoxia and control of the blood volume by regulation of thirst [153]. Elevated levels of AM have been observed in animals receiving exogenous endotoxin [154]. Few studies have examined AM levels in infection and sepsis [152,155-157]. These studies were characterized by few patients, heterogenic aetiology to SIRS and infection. These studies focused upon severe sepsis and septic shock [152,155-157].

VI. Study hypotheses and objectives

VI.1 Infections and sepsis in a department of internal medicine

The focus of the 3 cohorts in this Ph.D. study were patients admitted to a general department of internal medicine with suspected infections and sepsis. Most previous studies focusing on diagnostic, prognostic and immunological markers in sepsis have been conducted on intensive care unit populations, dominated by severe sepsis and septic shock (Figure 10). The patient population we focused on in our study was thus dominated by the milder end of the sepsis spectrum (Figure 10).

The main hypotheses in this study were:

1. PCT is a better diagnostic test marker for sepsis and infection compared to CRP in patients admitted to a general department of medicine.
2. Levels of PCT, LBP, IL-6, HMGB1 and sCD163 are associated to the severity and the presence of bacteremia in infection and sepsis.
3. PCT, LBP, IL-6, HMGB1 and sCD163 are prognostic markers in infection and sepsis.
4. Levels of sCD163 correlate to anti-inflammatory cytokines and levels of HMGB1 correlate to pro-inflammatory cytokines and other pro-inflammatory molecules.

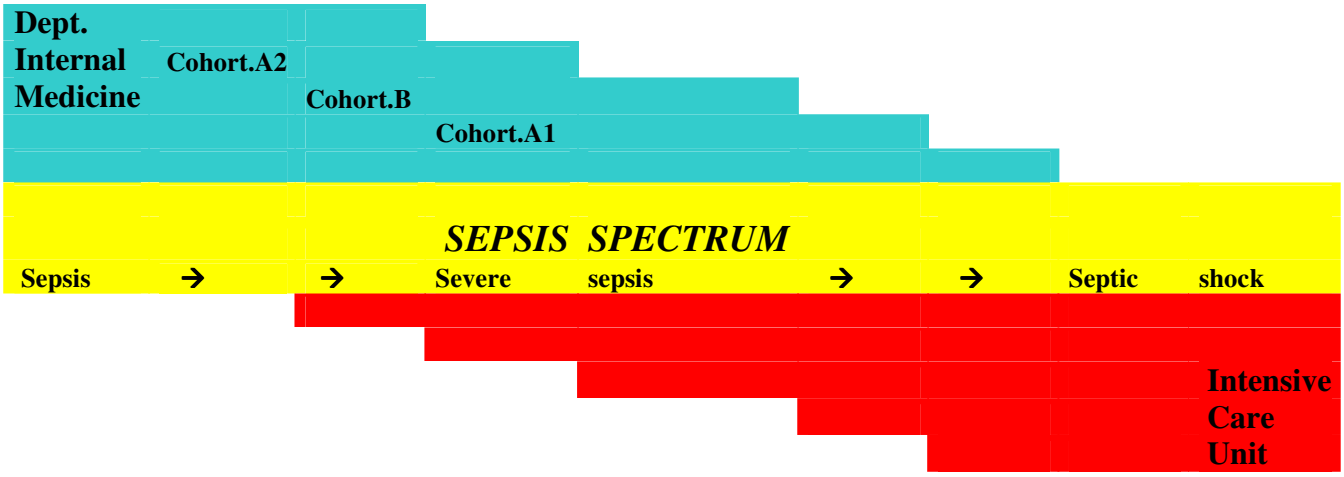


Figure 10
The sepsis spectrum in a general department of internal medicine compared to the intensive care unit

VI.2 Cohort A.1 ("suspected severe infections/sepsis") PAPER I

The objective of the study using cohort A.1 was to examine a panel of inflammatory markers in a cohort consisting of prospectively admitted patients with suspected severe infections and bacteremia in a department of internal medicine (Table 6 & Table 7). This cohort represented the more severe spectrum of sepsis in a general department of medicine (Figure 10).

VI.3 Cohort A.2 ("suspected infection") PAPER II, III & IV

The objective of the study using cohort A.2 was to examine a panel of inflammatory markers and examine the diagnostic test abilities of PCT, LBP, CRP and IL-6 in a cohort consisting of prospectively admitted patients with suspected infections admitted to a department of internal medicine (Table 6 & Table 7). This cohort represented the less severe spectrum of sepsis in a general department of medicine (Figure 10).

VI.4 Cohort B ("patients with confirmed bacteraemia") PAPER V

The objective of the study using cohort B was to examine a panel of inflammatory markers in a cohort consisting of prospectively admitted patients with verified bacteremia admitted to a department of internal medicine (Table 6 & Table 7). This cohort represented a subgroup of patients who all had bacteremia at the time of inclusion.

Table 6

Measured inflammatory markers in different cohorts

Cohort:	HMGB1	sCD163	PCT	LBP	IL-6	IL-10	CRP	WBC	Neutro
A.1 "suspected severe infections/sepsis"	**X** Paper 1		**X** Paper 1	**X** Paper 1	**X** Paper 1		**X** Paper 1	**X** Paper 1	**X** Paper 1
A.2 "suspected infection"	**X** Paper 4	**X** Paper 3	**X** Paper 2	**X** Paper 2	**X** Paper 2		**X** Paper 2	**X** Paper 2	**X** Paper 2
B "confirmed bacteremia"	**X** Paper 5	**X** Paper 5	**X** Paper 5	**X** Paper 5	**X** Paper 5	**X** Paper 5	**X** Paper 5	**X** Paper 5	**X** Paper 5

HMGB1, High mobility group box-1 protein; sCD163, Soluble haemoglobin scavenger receptor; PCT, Procalcitonin; LBP, Lipopolysaccharide-binding protein; IL-6, Interleukin-6; IL-10, Interleukin-10, CRP, C-reactive protein; WBC, White blood cell count; Neutro, Neutrophils.

Table 7

Objectives in different cohorts

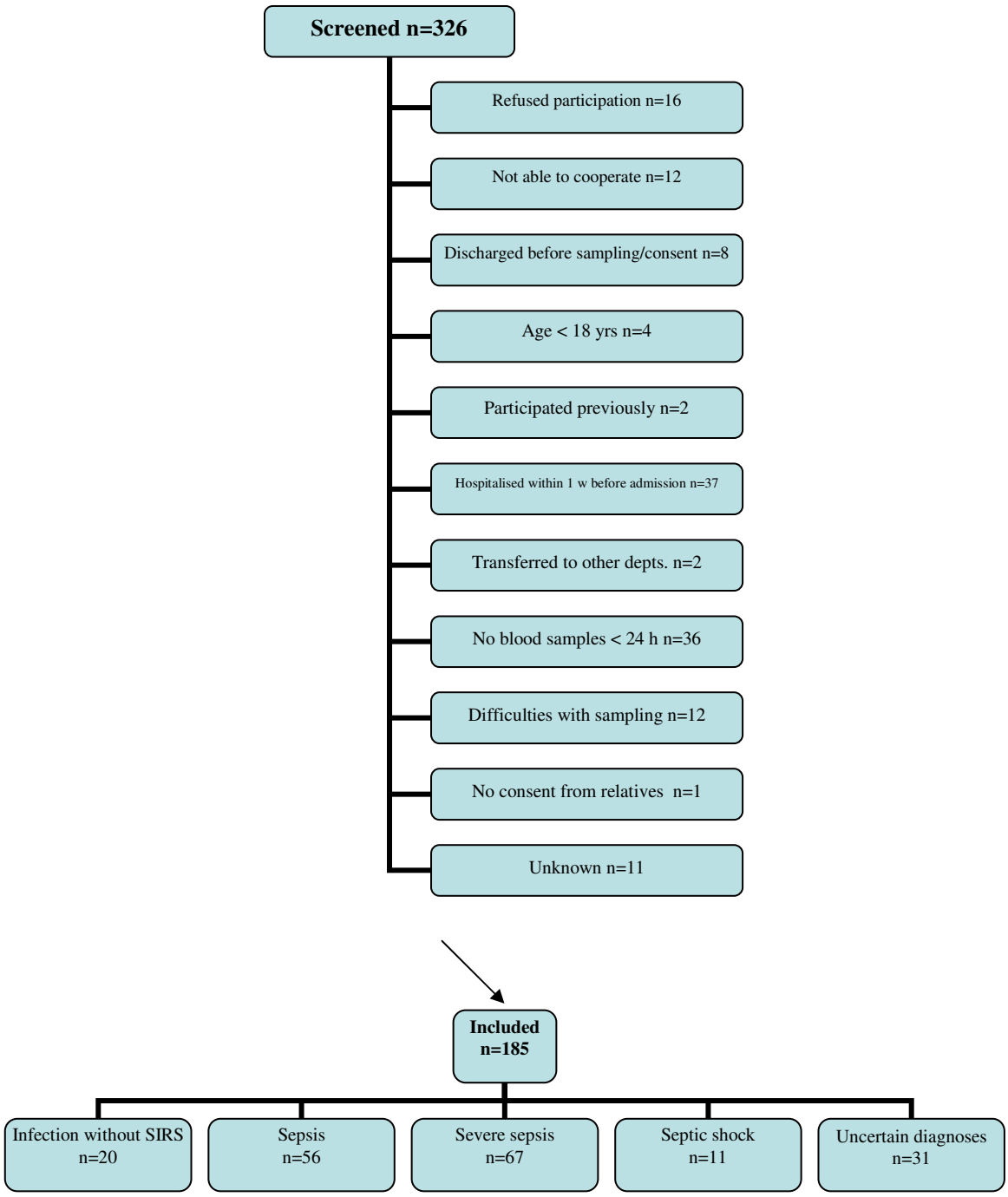
Cohort:	Diagnostic Test Study	Exploratory Study
A.1 "suspected severe infections/sepsis"		**X** Paper 1
A.2 "suspected infection"	**X** Paper 2	**X** Paper 3 & 4
B "confirmed bacteremia"		**X** Paper 5

VII. Patients, methods and study groups

VII.1 Patients and Setting

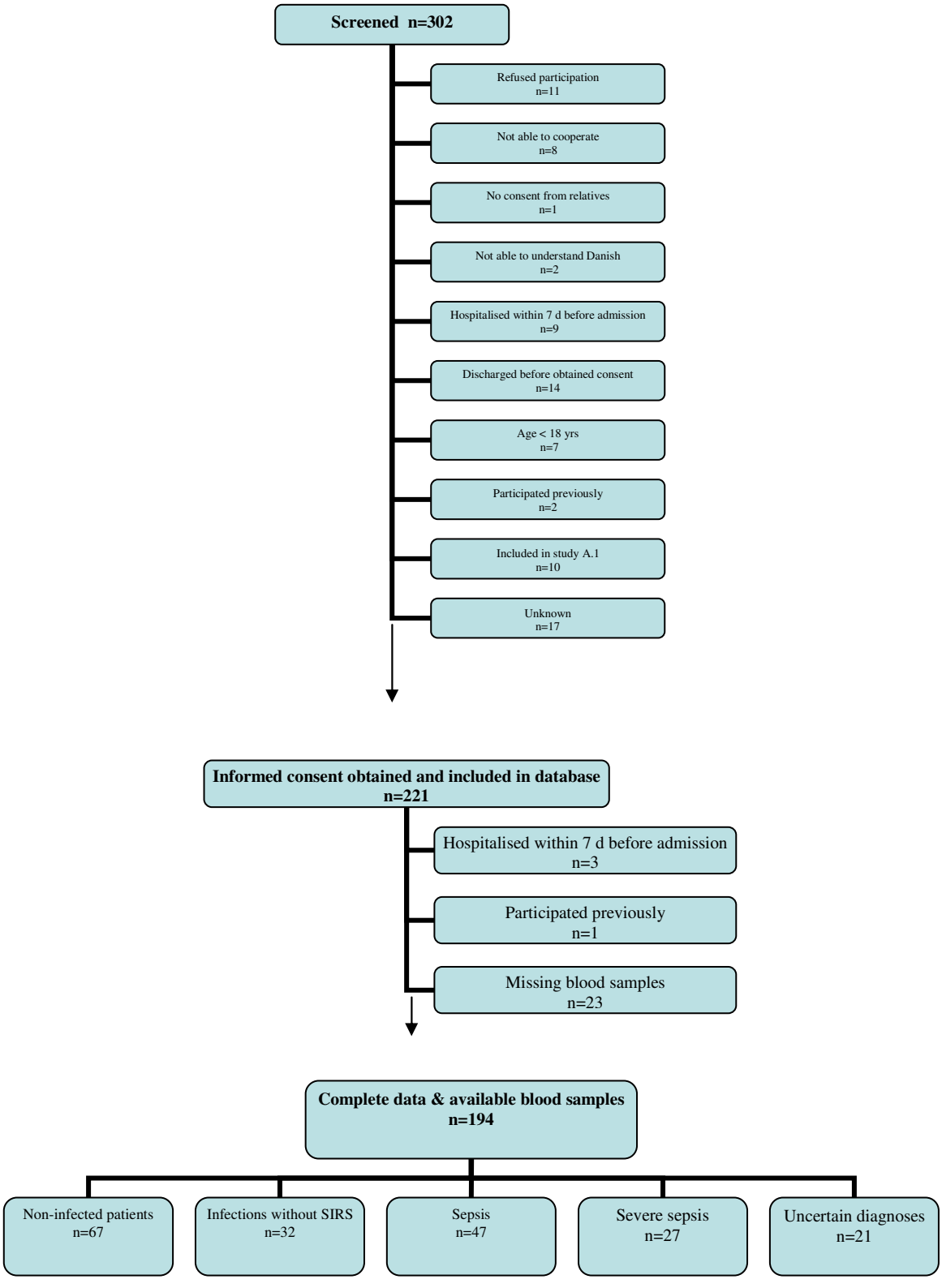
Cohort A.1 ("suspected severe infections/sepsis") PAPER I

1. Patients admitted to the department of Internal Medicine C at Odense University Hospital in the period January 2003 until June 2005 (with a break in the period May 2003 until December 2003).
2. Inclusion criteria:
    a. Suspicion of severe infection by the doctor in charge
    b. Start of sepsis treatment according to our sepsis guidelines with empirical antibiotics
    c. Inclusion and blood sampling should be possible within 24 h after admission
3. Exclusion criteria:
    a. Age < 18 yrs
    b. Earlier participation in the study
    c. Prior hospitalization within seven days before admission
4. Our sepsis guidelines included empirical antibiotic therapy with at least two different antibiotics covering a broad range of pathogens. This has until recently been the standard approach in Danish hospital settings treating *severe* infections. Danish clinicians have traditionally used this strategy only in suspected severe infections (severe sepsis, septic shock, severe pneumonia). Our department used typically combinations of the following antibiotics: cefuroxime+gentamicin, cefuroxime+gentamicin+metronidazole, G-penicillin+ciproflocaxin, G-penicillin+dicloxacillin. After our inclusion phase was terminated we have modified departmental guidelines to broad spectrum antibiotic strategies without gentamicin. Patients included with these inclusion criteria were representative of *the more severe end of the sepsis spectrum* in a general department of medicine.
5. Flowchart: p.40


Screened n=326
Refused participation n=16
Not able to cooperate n=12
Discharged before sampling/consent n=8
Age < 18 yrs n=4
Participated previously n=2
Hospitalised within 1 w before admission n=37
Transferred to other depts. n=2
No blood samples < 24 h n=36
Difficulties with sampling n=12
No consent from relatives n=1
Unknown n=11
Included n=185
Infection without SIRS n=20
Sepsis n=56
Severe sepsis n=67
Septic shock n=11
Uncertain diagnoses n=31

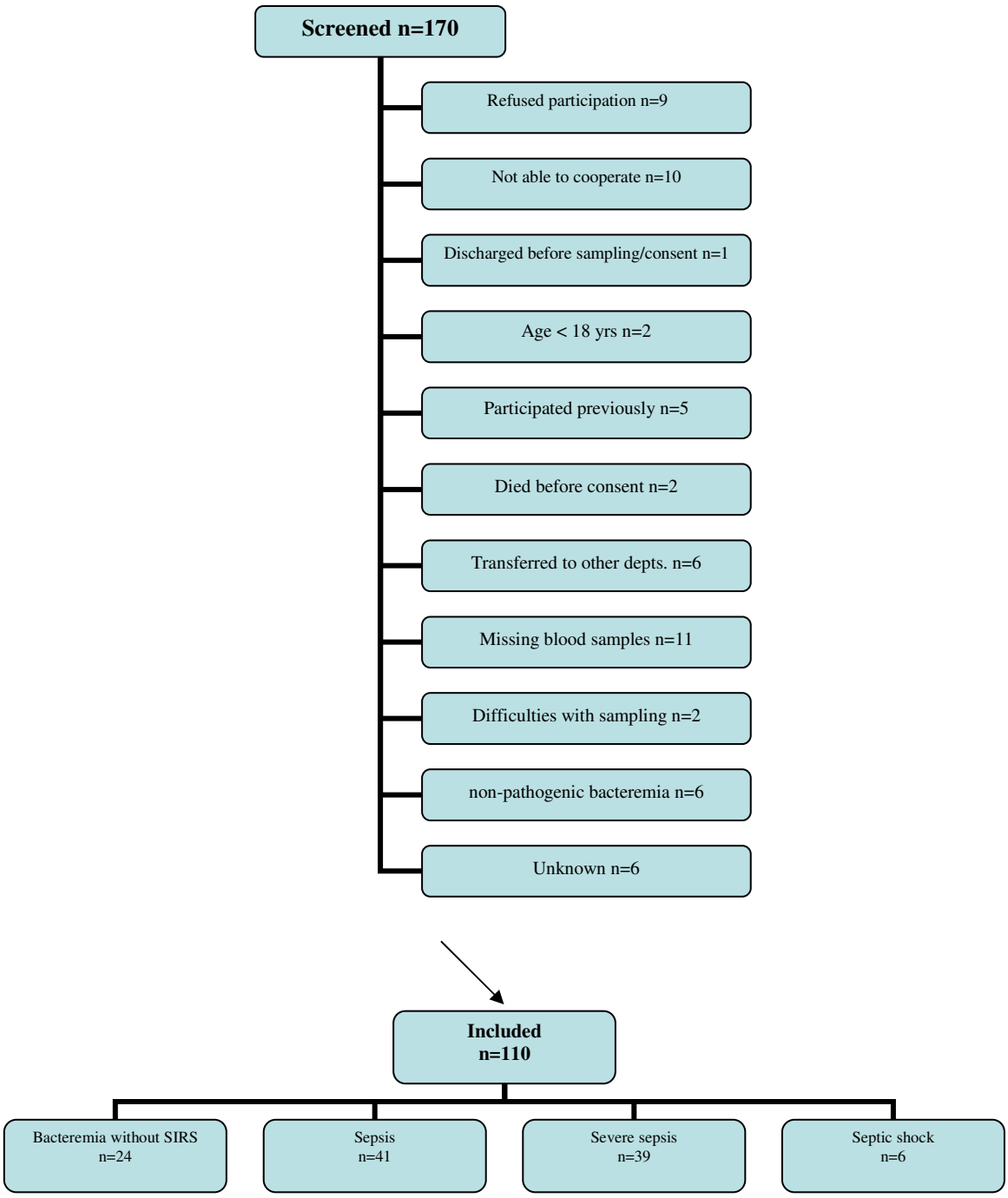
Cohort A.2 ("suspected infection") **PAPER II, III & IV**

1. Patients admitted to the department of Internal Medicine C at Odense University Hospital in the period: January 2003 until May 2003; Monday to Friday from 8 AM until 4 PM
2. Inclusion criteria:
    a. Suspected diagnosis of infection as judged by the referring physician
    b. Blood cultures drawn at the time of admission
3. Exclusion criteria:
    a. Age < 18 yrs
    b. Earlier participation in the study
    c. Prior hospitalization within seven days before admission
4. Blood cultures are standard procedures in our department when patients are admitted with a tentative diagnosis of infection and sepsis. The threshold for the clinician to order blood cultures is low. Patients included with these pragmatic inclusion criteria were representative of *the milder end of the sepsis spectrum* in a general department of medicine.
5. Flowchart: p. 42


Screened n=302
Refused participation n=11
Not able to cooperate n=8
No consent from relatives n=1
Not able to understand Danish n=2
Hospitalised within 7 d before admission n=9
Discharged before obtained consent n=14
Age < 18 yrs n=7
Participated previously n=2
Included in study A.1 n=10
Unknown n=17
Informed consent obtained and included in database n=221
Hospitalised within 7 d before admission n=3
Participated previously n=1
Missing blood samples n=23
Complete data & available blood samples n=194
Non-infected patients n=67
Infections without SIRS n=32
Sepsis n=47
Severe sepsis n=27
Uncertain diagnoses n=21

Cohort B ("patients with confirmed bacteraemia") **PAPER V**

1. Patients admitted to the department of Internal Medicine C at Odense University Hospital with verified bacteremia in the period November 2003 until June 2005
2. Inclusion criteria:
    a. Culture positive bacteremia verified by the Department of Clinical Microbiology
3. Exclusion criteria:
    a. Age < 18 yrs
    b. Earlier participation in the study
    c. Growth of a bacteria considered to be non-pathogenic
4. Study blood samples were drawn when the department of microbiology reported positive blood cultures. Because of the time delay of culturing and processing of the blood cultures in the department of clinical microbiology this could be 1-4 days after that blood cultures were drawn.
5. Flowchart: p. 44


Screened n=170
Refused participation n=9
Not able to cooperate n=10
Discharged before sampling/consent n=1
Age < 18 yrs n=2
Participated previously n=5
Died before consent n=2
Transferred to other depts. n=6
Missing blood samples n=11
Difficulties with sampling n=2
non-pathogenic bacteremia n=6
Unknown n=6
Included
n=110
Bacteremia without SIRS
n=24
Sepsis
n=41
Severe sepsis
n=39
Septic shock
n=6

VII.2 Laboratory assays

HMGB1

1. Technology: ELISA
2. Producer: Shino-Test Corporation, Tokyo, Japan
3. Laboratory: Department of Clinical Biochemistry, Aarhus University Hospital NBG
4. Reference: Yamada and colleagues [158]

sCD163

1. Technology: ELISA
2. Producer: in-house
3. Laboratory: Department of Clinical Biochemistry, Aarhus University Hospital NBG
4. Reference: Moller and colleagues [159]

PCT

1. Technology: Time-resolved amplified cryptate emission (TRACE) technology assay (Kryptor PCT®)
2. Producer: BRAHMS Aktiengesellschaft, Hennigsdorf, Germany
3. Laboratory: Department of Clinical Biochemistry, Sønderborg Hospital

LBP

1. Technology: chemiluminiscent immunometric assay (Immulite-1000®)
2. Producer: Diagnostic Product Corporation, Los Angeles, CA, USA
3. Laboratory: Department of Clinical Biochemistry, Sønderborg Hospital

IL-6

1. Technology: chemiluminiscent immunometric assay (Immulite-1000®)
2. Producer: Diagnostic Product Corporation, Los Angeles, CA, USA
3. Laboratory: Department of Clinical Biochemistry, Sønderborg Hospital

IL-10

1. Technology: chemiluminiscent immunometric assay (Immulite-1000®)
2. Producer: Diagnostic Product Corporation, Los Angeles, CA, USA
3. Laboratory: Department of Clinical Biochemistry, Sønderborg Hospital

CRP

1. Technology: Immunoturbidimetric assay (Modular P®)
2. Producer: Roche Ltd, Switzerland
3. Laboratory: Department of Clinical Biochemistry, Odense University Hospital

WBC and Neutrophils

1. Technology: Sysmex SE 9000®
2. Producer: TOA Corporation, Kobe, Japan
3. Laboratory: Department of Clinical Biochemistry, Odense University Hospital

VII.3 Statistics

Sample size

Cohort A.1 ("suspected severe infections/sepsis")

48 patients in each group were required for the unpaired t-test to have a 90% chance of detecting a difference in means of 10 ng/ml in PCT levels (Standard deviation: 15 ng/ml) between infected patients without bacteremia and bacteremic patients at the 5% level of significance (α=0.05, β=0.90).

Cohort A.2 ("suspected infection")

60 patients in each group were required for the unpaired t-test to have a 90% chance of detecting a difference in means of 0.4 ng/ml in PCT levels (Standard deviation: 0.6 ng/ml) between non-infected patients and infected patients at the 1% level of significance (α=0.01, β=0.90).

Cohort B ("patients with confirmed bacteraemia")

23 patients in each group were required for the unpaired t-test to have a 90% chance of detecting a difference in means of 5 mg/l in sCD163 levels (Standard deviation: 5 mg/l) between survivors and fatal cases at the 5% level of significance (α=0.05, β=0.90).

Statistical calculations

Data are presented with means ± standard deviations, medians and interquartile ranges in tables and box-plots. Correlations examined with Spearman´s rank correlation test. Significance testing performed with the Kruskall-Wallis test and the Wilcoxon two-sample test. P-values of less than 0.05 were considered as statistically significant. The abilities of the studied markers in identifying bacteremic patients were studied by comparing the area under the curve and by comparing the specificities when the sensitivity was approximately 80%.

VII.4 Strengths, weaknesses and biases in study design

Strengths

1. The study focused on the sepsis spectrum seen in a department of internal medicine
2. The patient sample was representative for the large majority of infected patients treated on medical non-ICU units
3. The study was composed of 3 cohorts reflecting the whole spectrum of infection/sepsis on departments of internal medicine
4. The study had a prospective design and pre-set definitions on the presence of infection, severity and co-morbidity were used.
5. PCT, LBP, IL-6 and IL-10 were measured with sensitive automatised fast assay systems
6. Two of the cohorts (A.1 and A.2) used broad inclusion criteria reducing the risk of spectrum bias and promoting inclusion of the whole spectrum of infections with and without SIRS

Weaknesses

1. Heterogeneous population of patients
2. Heavy burden of co-morbidity
3. Different length of disease prior to inclusion in the study
4. Single point measurements of the studied inflammatory markers
5. Single physician was responsible for classification of the patients and input into database

Possible biases in study design

1. Work-up bias: Research physician was blinded to all inflammatory laboratory results in the process of classifying and data-processing of all included patients. The database was closed before any analyses of the data. Laboratory technicians were blinded from all clinical data.
2. Spectrum bias: Broad inclusion criteria were used to minimise spectrum bias. In studies focusing on severe disease like sepsis, included patients will often be in the milder end of the disease spectrum. The collection of informed consent can be difficult in the most ill

patients because patients can be comatose, relatives in crisis and rapid fatal outcome before the possibility of asking for informed consent.

3. Imperfect gold-standard bias: Infection and sepsis can be difficult to document with microbiological data in up to 40% of sepsis cases. This makes imperfect gold-standard bias an important issue in studies focusing on infections and sepsis. This can be avoided by excluding all patients without microbiological evidence of infection and this would tend to increase the efficacy of studied diagnostic test markers. However such a study design will not be representative of the clinical reality that faces clinicians diagnosing and treating infections/sepsis. The gold-standard of infection in sepsis studies will thus often be a compromise between microbiological evidence of infection and more pragmatic definitions of infection. This compromise will have the potential of introducing imperfect gold-standard bias. However if the studied test and the imperfect gold-standard are independent it can be expected that the sensitivity and specificity of a studied diagnostic test will be underestimated. We consider an underestimation of the studied test preferable to an overestimation due to exclusion of all patients without hard evidence for infection. One of our studies (Cohort B) focused only on patients with significant bacteremia and therefore had a robust gold-standard for infection.

VIII. Results

VIII.1 Results: Cohort A.1 (Paper 1)

1. HMGB1, LBP, PCT, IL-6 and CRP were elevated in infected patients compared to healthy controls in a cohort of patients dominated by severe sepsis and bacteremia in a department of internal medicine

2. The studied inflammatory markers were not prognostic for fatal outcome in this cohort

3. PCT performed best as a diagnostic test marker discriminating between bacteremic and non-bacteremic patients in this cohort (Figure 11)

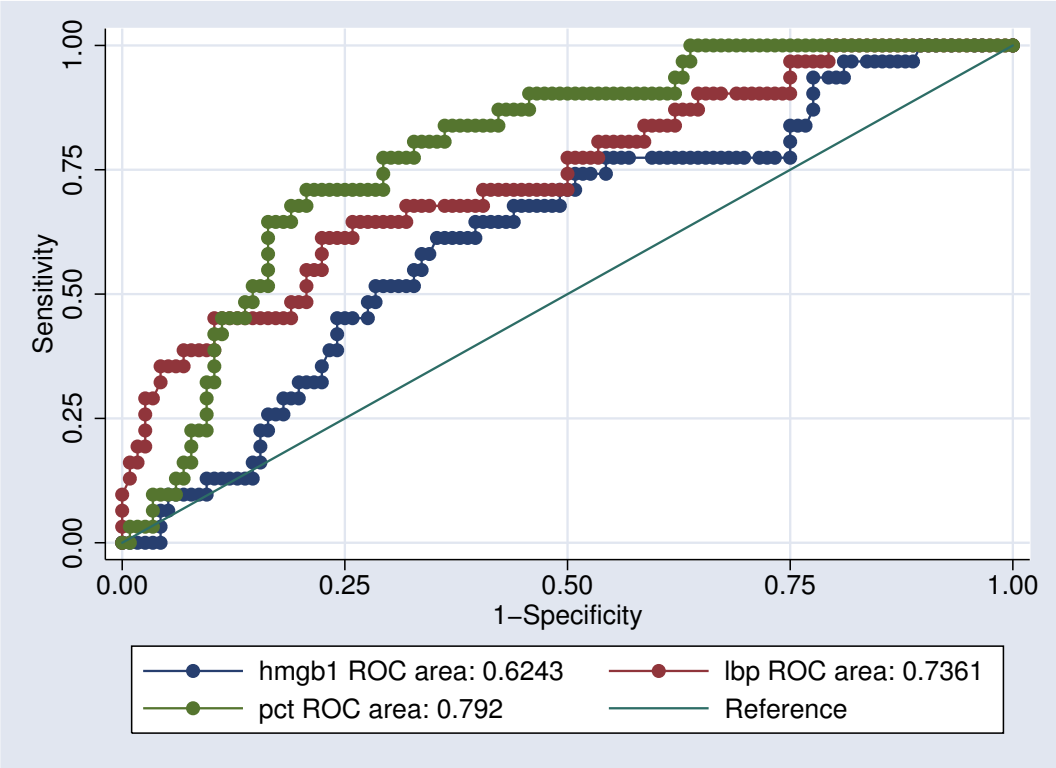
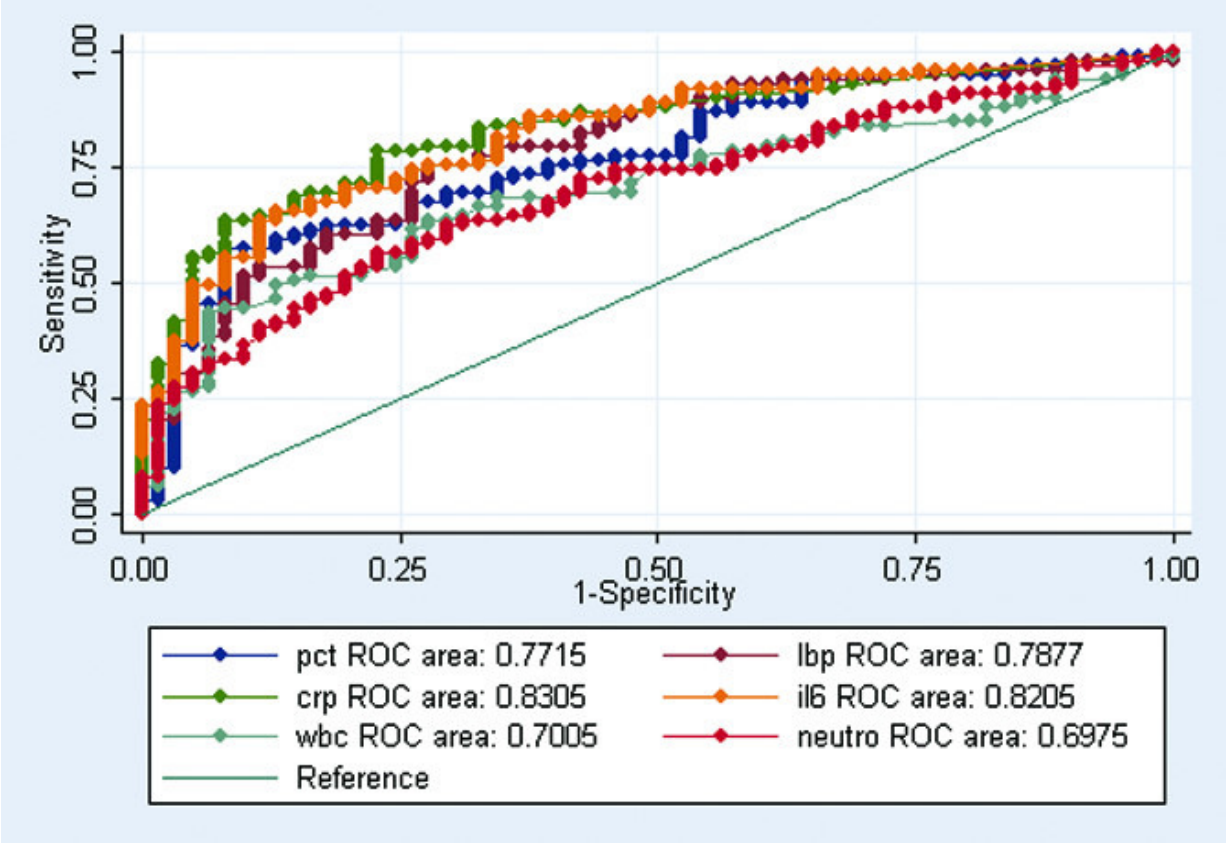


Figure 11
ROC curve comparing HMGB1, LBP and PCT discriminating abilities between bacteremic and non-bacteremic patients (P<0.05)

VIII.2 Results: Cohort A.2 (Paper 2, 3 & 4)

1. Paper 2: CRP, LBP and IL-6 were superior to PCT as diagnostic test markers for the presence of infection and sepsis in a cohort of community-acquired infections/sepsis in the milder end of the sepsis spectrum admitted to a department of internal medicine (Figure 12)



ROC curves comparing inflammatory markers discriminating abilities between noninfected patients and all infected patients (P < 0.05). Receiver-operating characteristic (ROC) curves comparing procalcitonin (pct), lipopolysaccharide-binding protein (lbp), C-reactive protein (crp), IL-6 (il6), white blood cell (wbc) and neutrophil (neutro) discriminating abilities between noninfected patients and all infected patients ($P < 0.05$).

Figure 12

2. Paper 3: Levels of sCD163 were higher only in patients with severe sepsis and/or bacteremia in this cohort. sCD163 did not discriminate between non-infected and infected patients (Figure 13 & Figure 14)

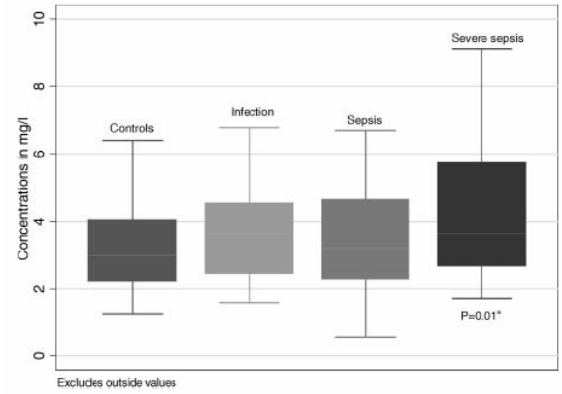


Fig. 1. sCD163 levels in the non-infected group (controls), infection without SIRS (infection), sepsis and severe sepsis. Boxplot: white lines denote median values, boxes represent 25th to 75th percentile, and whiskers indicate range. [a]p-value calculated with Wilcoxon's two-sample test (compared to non-infected control group).

Figure 13

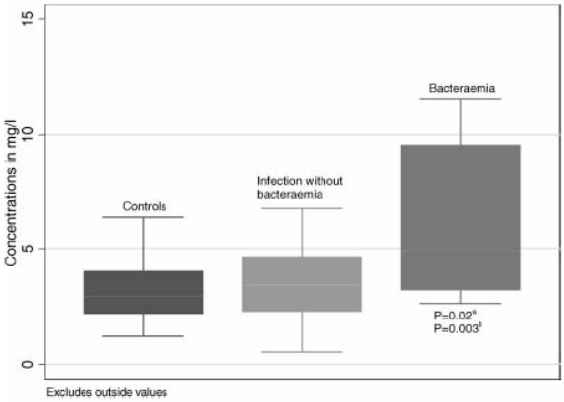
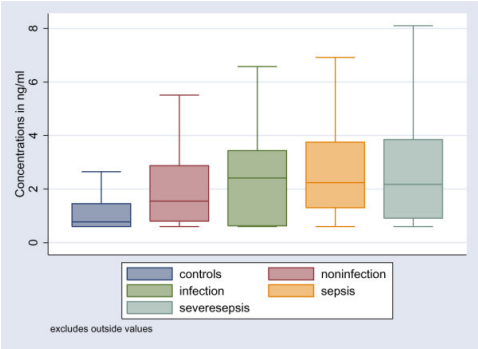


Fig. 2. sCD163 levels in the non-infected group (controls), infected patients without bacteraemia, and patients with bacteraemia. Boxplot: white lines denote median values, boxes represent 25th to 75th percentile, and whiskers indicate range. [a]p-value calculated using Wilcoxon's two-sample test (compared to infected patients without bacteraemia); [b]p-value calculated using Wilcoxon's two- sample test (compared to non-infected control group).

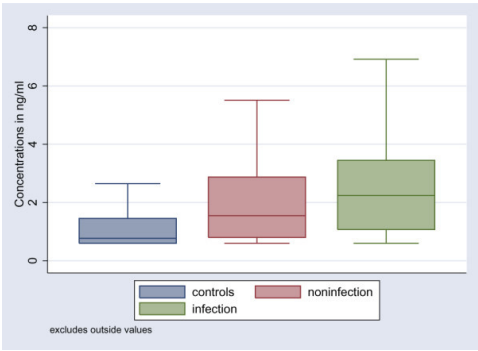
Figure 14

3. Paper 4: Levels of HMGB1 were higher among infected patients compared to healthy controls in this cohort. HMGB1 did not discriminate between non-infected and infected patients (Figure 15, Figure 16 & Figure 17)



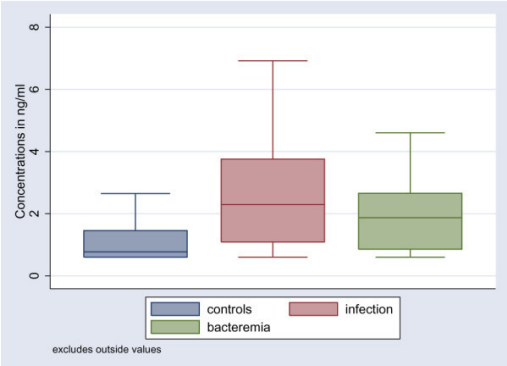
Boxplot of HMGB1 levels in healthy controls, non-infected patients ($P < 0.001$ compared to healthy controls), infected patients without systemic inflammatory response syndrome (SIRS) ($P = 0.32$ compared to non-infected patients), patients with sepsis ($P = 0.48$ compared to infected patients without SIRS), and patients with severe sepsis ($P = 0.37$ compared to patients with sepsis). HMGB1, high mobility group box-1 protein.

Figure 15



Boxplot of HMGB1 levels in healthy controls, non-infected patients ($P < 0.001$ compared to healthy controls), and all infected patients ($P = 0.054$ compared to non-infected patients). HMGB1, high mobility group box-1 protein.

Figure 16



Boxplot of HMGB1 levels in healthy controls, infected patients without bacteraemia ($P < 0.0001$ compared to healthy controls), and patients with bacteraemia ($P < 0.05$ compared to healthy controls; $P = 0.38$ compared to infected patients without bacteraemia). HMGB1, high mobility group box-1 protein.

Figure 17

VIII.3 Results: Cohort B (Paper 5)

1. Levels of HMGB1, sCD163, PCT, LBP, IL-6 and IL-10 were elevated in bacteremic patients compared to healthy controls (Figure 18 & Figure 19)

2. sCD163 and IL-6 were prognostic markers in this cohort with bacteremic patients on a department of internal medicine

3. HMGB1 correlated to pro-inflammatory markers while sCD163 correlated to the anti-inflammatory marker IL-10 and to IL-6

4. There was no statistically significant difference regarding HMGB1, sCD163 and LBP levels between gram-negative bacteremia and gram-positive bacteremia. Significantly lower levels of PCT were observed in *S. aureus* bacteremia compared to *S. pneumoniae* bacteremia (Figure 20-23)

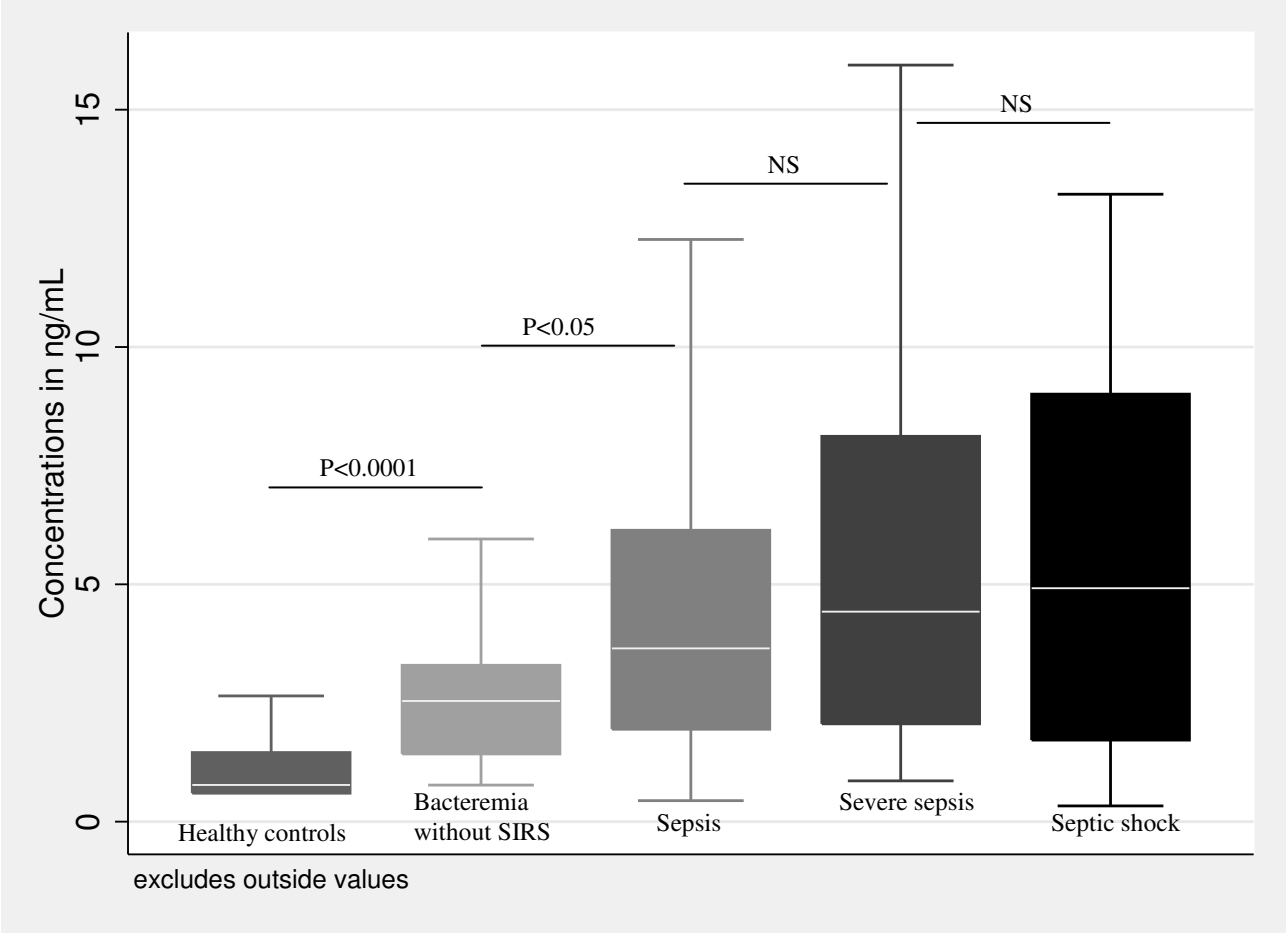


Figure 18: HMGB1 levels among bacteremic patients with different disease severity

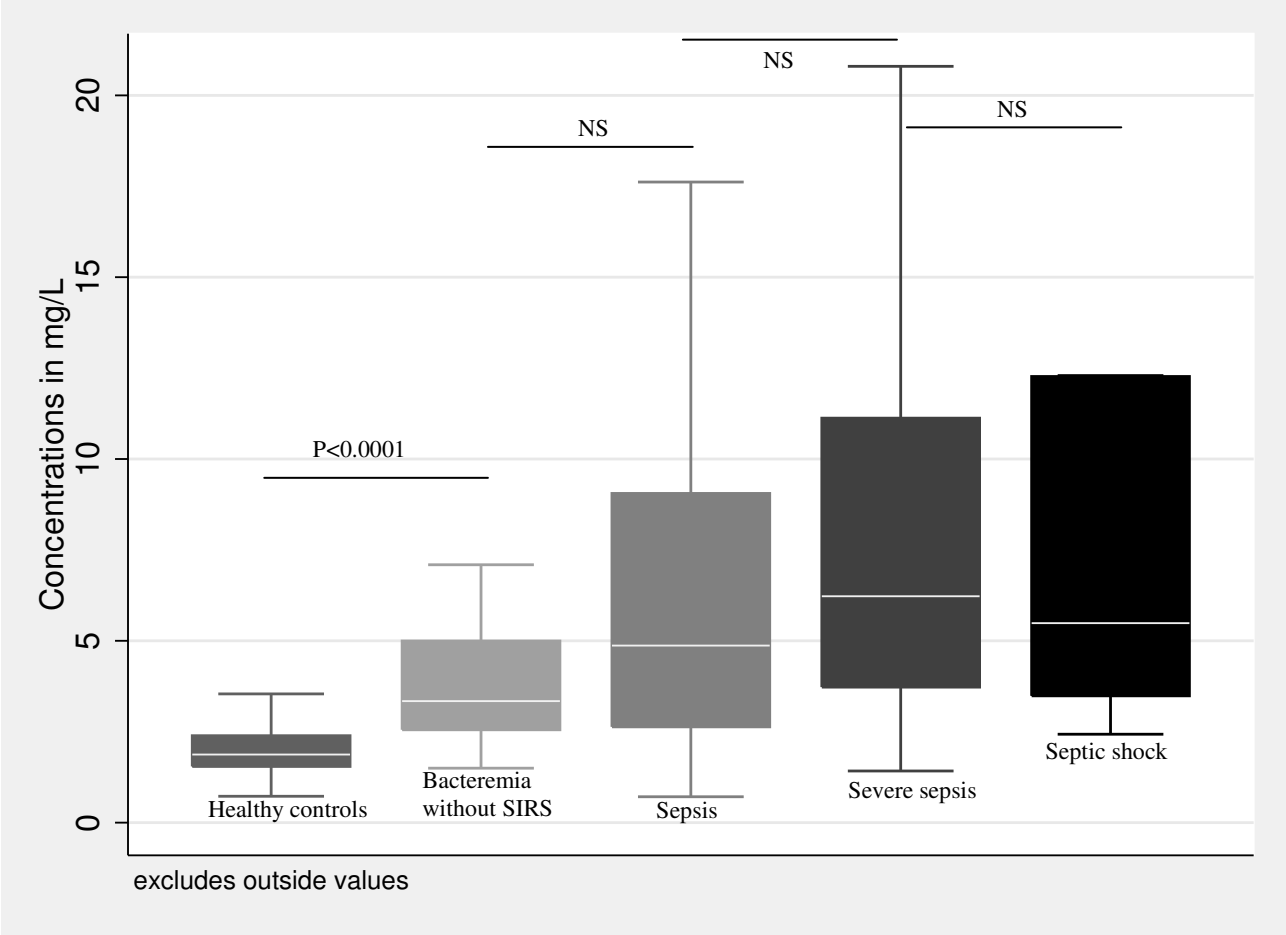


Figure 19: sCD163 levels among bacteremic patients with different disease severity

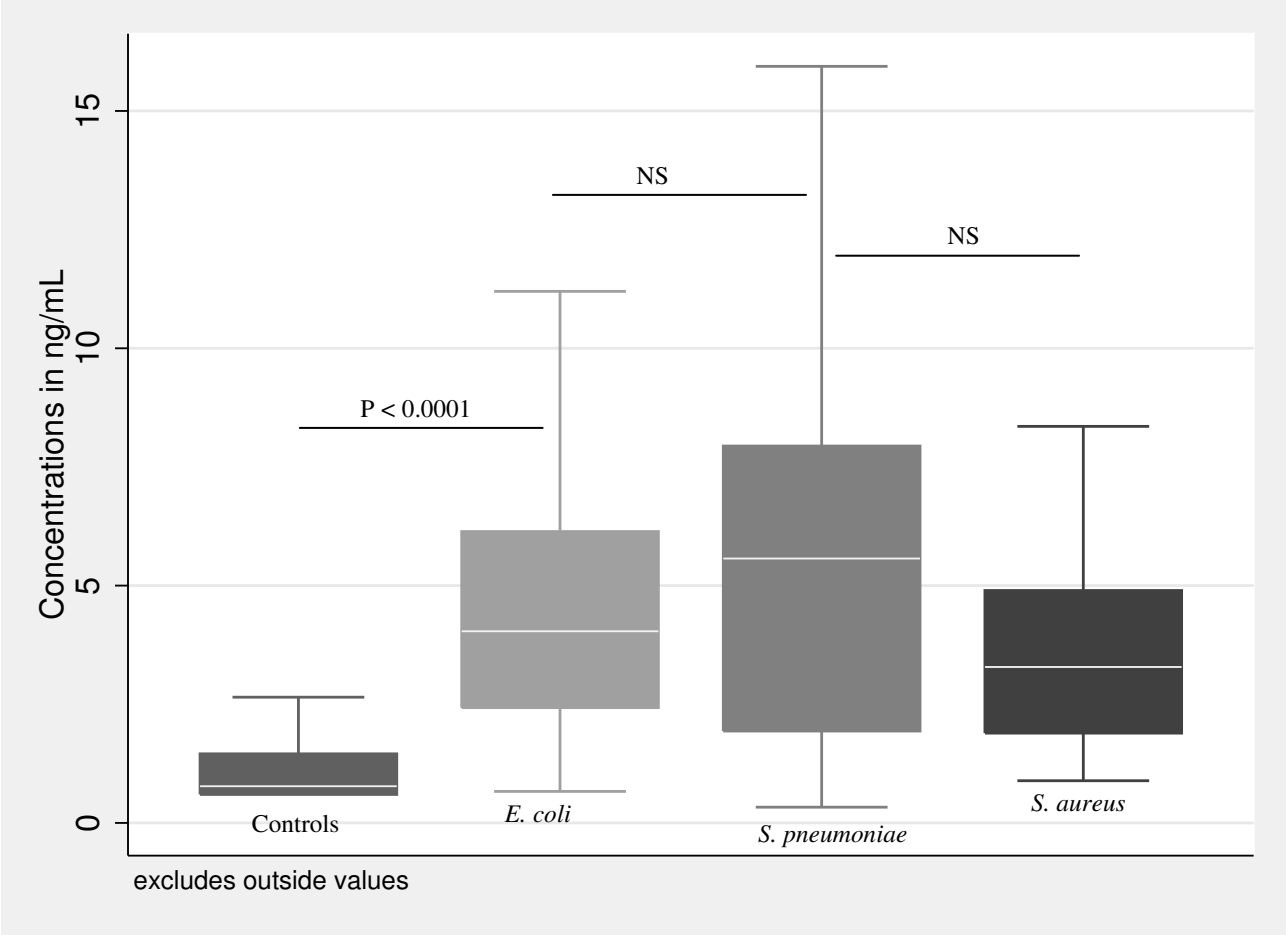


Figure 20: HMGB1 levels in healthy controls, bacteremia with *E. coli*, bacteremia with *S. pneumoniae* and bacteremia with *S. aureus* (Kruskal-Wallis test: P < 0.001).

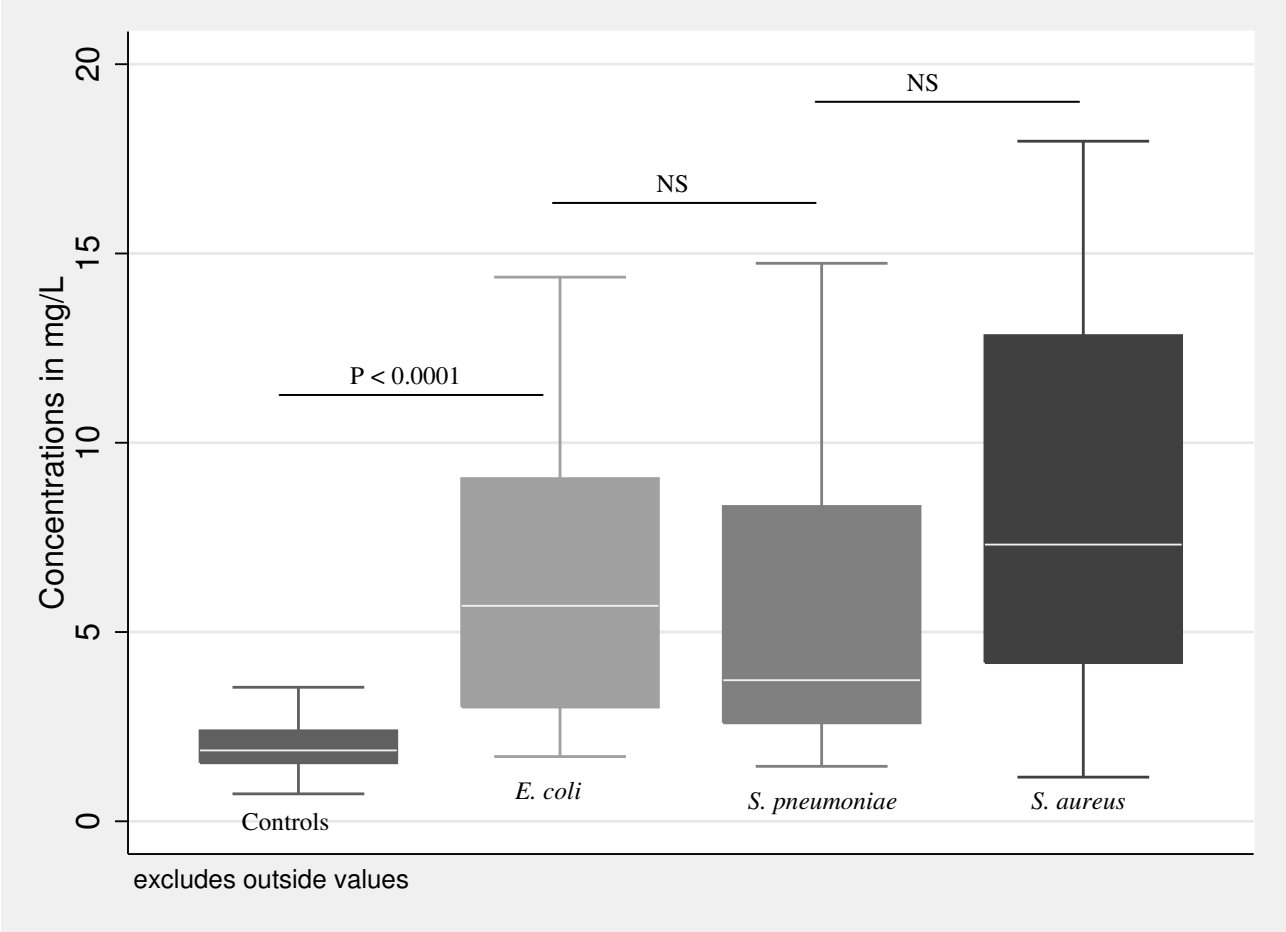


Figure 21: sCD163 levels in healthy controls, bacteremia with *E. coli*, bacteremia with *S. pneumoniae* and bacteremia with *S. aureus* (Kruskal-Wallis test: P < 0.001).

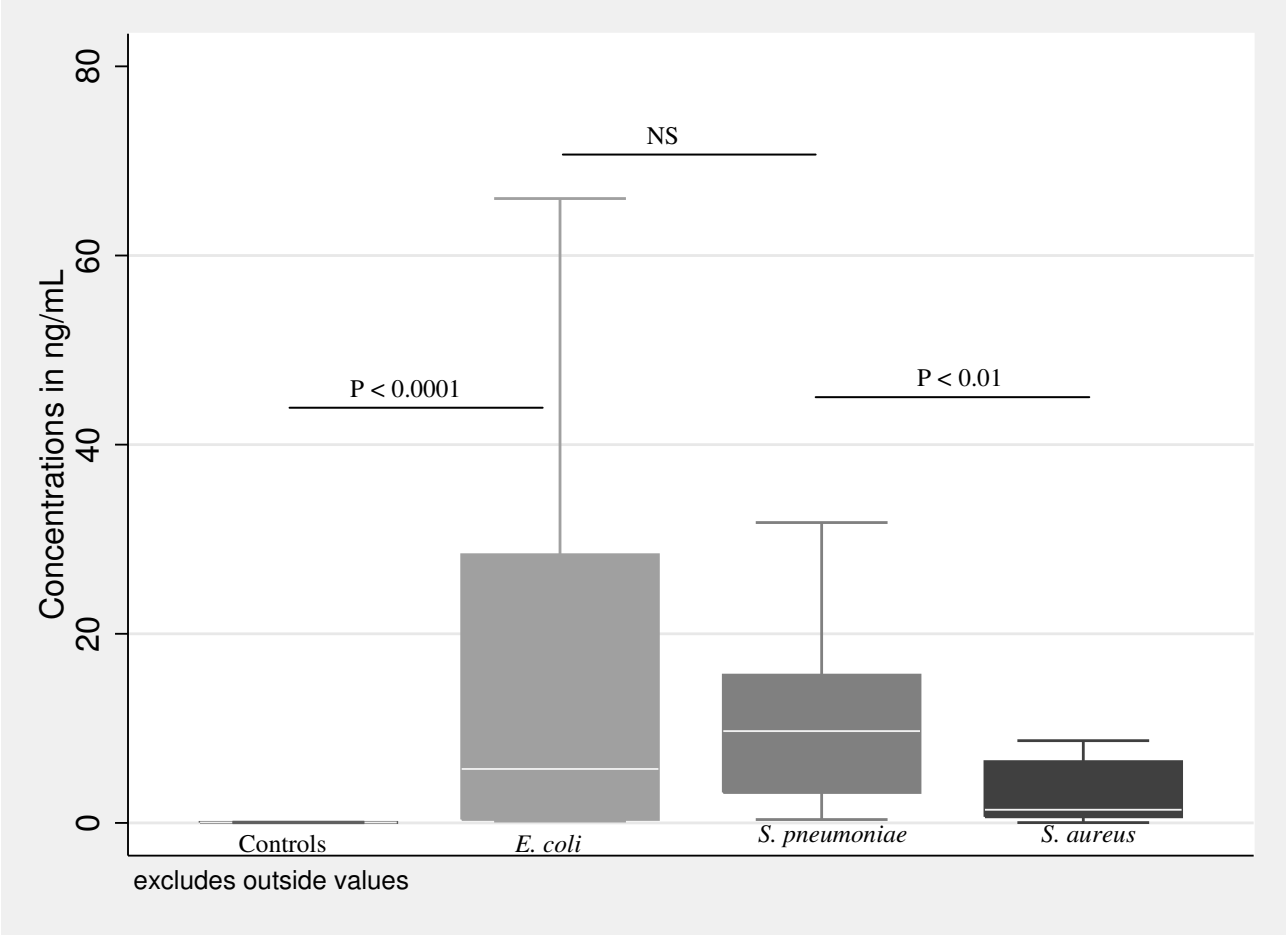


Figure 22: PCT levels in healthy controls, bacteremia with *E. coli*, bacteremia with *S. pneumoniae* and bacteremia with *S. aureus* (Kruskal-Wallis test: P < 0.001).

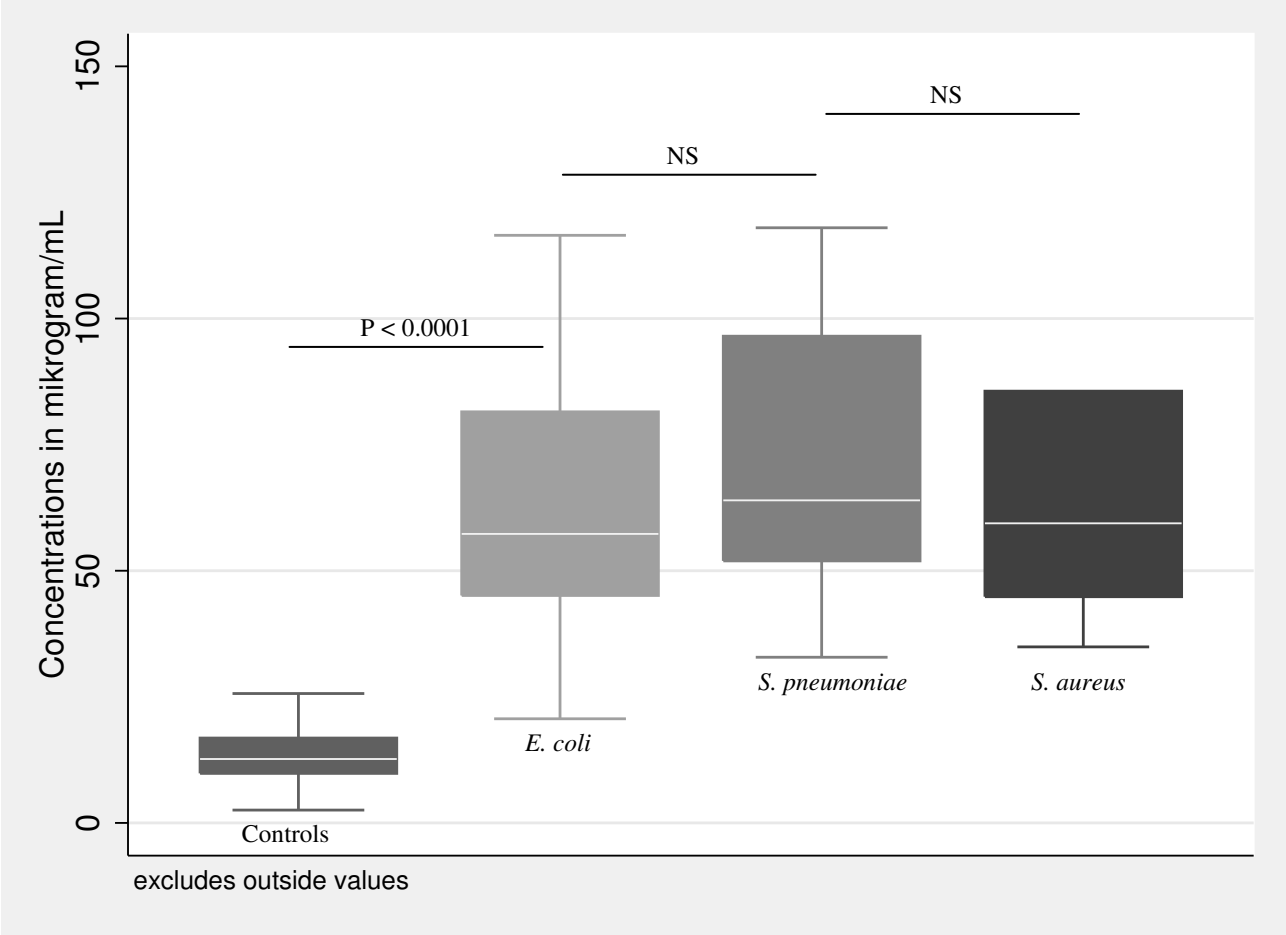


Figure 23: LBP levels in healthy controls, bacteremia with *E. coli*, bacteremia with *S. pneumoniae* and bacteremia with *S. aureus* (Kruskal-Wallis test: P < 0.001).

VIII.4 Discussion

A few studies have previously been conducted focusing on the diagnostic test abilities of PCT in diagnosing infection in emergency departments and medical departments [86-92]. Six of these studies had fewer patients with the target condition compared to our study (Paper 2) [86-91]. The AUC of PCT in distinguishing between non-infected and infected patients in 4 of these studies were between 0.69 and 0.79 [87,88,91,92]. Two of these studies reported the AUC's of CRP in distinguishing between the abovementioned groups of patients [88,91]. The AUC's of CRP were 0.88 [88] and 0.81 [91] in these two studies. One large study did not report the AUC of CRP [92]. Our results (Paper 2) confirm the abovementioned findings [88,91], suggesting CRP to be a better marker than PCT in diagnosing infection in emergency and medical departments. Our data confirms that PCT is associated to the presence of bacteremia (Paper 1 and 5). This has been shown previously in other studies [100-107]. Our study data also confirms that PCT is a severity marker of sepsis (Paper 1, 2 and 5). This has also been shown previously [80,88].

Only two diagnostic test studies regarding LBP as a diagnostic test marker for infection in adult patients have been published previously [117,118]. These studies focused on febrile neutropenia and ICU patients. Our data are to our knowledge the first published results on diagnostic test abilities in adults with infection and sepsis (Paper 2). Our data suggested that LBP performed equally to CRP as a diagnostic test marker for the presence of infection in the department of internal medicine (Paper 2). LBP was found to be a severity marker (Paper 1, 2 and 5).

IL-6 has previously been shown to be a prognostic marker in severe infections [143-145]. IL-6 was found to be a prognostic marker in our bacteremia cohort (Paper 5).

Only one previous study has been published regarding the diagnostic test abilities of sCD163 [160]. This study focused on the abilities of sCD163 in diagnosing bacterial meningitis (AUC: 0.72) and bacterial systemic infections (AUC: 0.83). However, the SIRS criteria were not used in this study. sCD163 performed poorly in our study as a diagnostic test marker for infection (AUC: 0.59) (Paper 3). Our data showed that sCD163 was a prognostic marker in bacteremic patients (Paper 5). This has been shown previously in a cohort of pneumococcal bacteremia [133].

Elevated levels of HMGB1 have been observed in previous studies focusing on sepsis

[134,136,137,138,139]. Our study data showed elevated levels of HMGB1 in patients with infection and sepsis compared to healthy controls (Paper 1, Paper 5). HMGB1 performed poorly as a diagnostic test marker for infection with an AUC of only 0.59 (Paper 4).

IX. Conclusions

CRP, LBP and IL-6 were superior to PCT as diagnostic test markers for the presence of infection and sepsis in medical patients in a medical department. Levels of PCT were much lower among patients with infections and sepsis on medical departments compared to previously reported PCT data from ICU units. PCT was not associated to the prognosis of the included patients with infections without SIRS, sepsis and bacteremia. PCT was a severity marker in sepsis. PCT and IL-6 were superior to CRP, LBP and HMGB1 as diagnostic test markers for the presence of bacteremia. Much higher levels of PCT were seen among bacteremic patients compared to infected non-bacteremic patients. PCT was a marker for bacteremia and severity in sepsis on medical departments.

LBP was a severity marker in sepsis. High levels of LBP were seen in patients with infections without SIRS, sepsis and bacteremia on medical departments. LBP performed equally to CRP and IL-6 as diagnostic test markers of infection in medical patients on medical departments. High levels of LBP were seen both in gram-negative and gram-positive bacteremia. LBP was not associated to the prognosis of the included patients with infections without SIRS, sepsis and bacteremia.

sCD163 and IL-6 were prognostic markers in patients with bacteremia. sCD163 correlated to the anti-inflammatory cytokines IL-6 and IL-10 suggesting sCD163 to be an anti-inflammatory mediator in severe infections. sCD163 did not correlate to measured pro-inflammatory markers except from a weak correlation to the neutrophil count. sCD163 levels were elevated among medical patients with severe sepsis and with bacteremia. sCD163 performed poorly in discriminating between non-infected and infected patients.

HMGB1 correlated to the measured pro-inflammatory markers but not to sCD163, IL-6 and IL-10, suggesting a pro-inflammatory role for HMGB1. HMGB1 levels were elevated in infected patients compared to a healthy control group. HMGB1 performed poorly in discriminating between non-infected and infected patients. HMGB1 was not associated to the prognosis of the included patients with infections without SIRS, sepsis and bacteremia.

X. Perspectives

The perspective of the studies conducted in this thesis and many other studies focusing on diagnostic and immunological markers in sepsis are the possibility of developing a panel of diagnostic, prognostic and "immune status" (pro-inflammation, anti-inflammation, homeostasis) markers for each single patient with sepsis. This could maybe in the future stratify sepsis patients to individualised treatment strategies including immuno-modulating treatments. Diagnostic candidate markers for the presence of infection could be CRP, LBP and IL-6. PCT could have a role in distinguishing between viral and bacterial sepsis and a role as a severity marker. CRP, LBP and PCT could have a role in monitoring treatment response in sepsis with consecutive measurements. Possible future prognostic markers could be sCD163 and IL-6. Possible markers for the immune monitoring in sepsis could maybe be HMGB1 and sCD163 for pro-inflammation and anti-inflammation respectively.

Studies focusing on consecutive measurements of PCT, CRP, LBP, IL-6, HMGB1 and sCD163 on infected medical cohorts should be encouraged. The benefit of consecutive measurements should be examined: diagnostic abilities, prognostic abilities, abilities to monitor treatment response. We are conducting two studies looking upon these aspects.

Other studies should be carried out examining if some of our study markers could be used to stratify patients to strategies involving for instance earlier access to intermediate/intensive care treatment and to faster molecular microbiological diagnostic techniques to identify the involved pathogen species in patients with sepsis. This could have the potential of improving survival both by earlier supportive therapy and earlier correct choice of antibiotics covering the identified pathogen. We are planning a possible study focusing on these aspects.

New translational studies should be encouraged looking upon the role of CD163 and sCD163 in infection and sepsis. We are planning a possible study focusing on several aspects of haemoglobin metabolism, inflammation and haemophagocytic syndrome in infection and sepsis.

Continued efforts should also be put on developing a golden standard for immune status in sepsis research. This could have the potential to be a gold-standard that potential biomarkers in sepsis could be benchmarked against. Increased insight in the immunopathogenesis of sepsis would offer

the potential to generate new diagnostic and treatment options in sepsis.

XI. Reference list

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XII. Paper 1

XVII. Appendices

XVII.1 Evaluation of a commercial HMGB1 ELISA kit

Serum – HMGB1

Synonyms: High mobility group box protein 1; amphoterin

Validation of ELISA-kit

Period: May 2006 – August 2006
Location: Department of Clinical Biochemistry, Aarhus University Hospital, NBG, Denmark
Responsible: Holger Jon Møller, MD, PhD, Kirsten Bank Petersen, Labtech.

Problem formulation

To validate a new ELISA kit for measurement of HMGB1 in serum.

Conclusion

The kit is well suited for the purpose.
For research purposes precision is adequate (even without duplicate measurements).

Purpose

To measure HMGB1 in sera from healthy and patients with infection for research purposes

Quality requirements

No known guidelines.
The biological variation of HMGB1 is not known.
Precision requirements arbitrarily set to < 10% (intra-assay) and < 15 % interassay.

Method

Two step ELISA. Polyclonal antiHMGB1 coated. Peroxidase-labelled secondary monoclonal antibody. TMB substrate.

Instrument

Manually performed. Routine ELISA reader.

Reagents

HMGB1 ELISA kit, Shino-Test corporation.

Calibrator

Pig HMGB1. Supplied with kit. >98 % sequence homology with human HMGB1.
Two point calibration each run. Linear interpolation (point to point through zero).

Controls

Internal control:
Control 1 (level 15-35 ng/ml) supplied with kit.
Control 2 (level 8-12 ng/ml) serum-pool produced locally (>50 aliquots frozen).
Both controls in duplicate each run.
External control system:
None exists

Clinical decision limits
Reference range not established.
Sex- and age- variability not known.
Biological variation not known.
Level in pathological conditions not known.

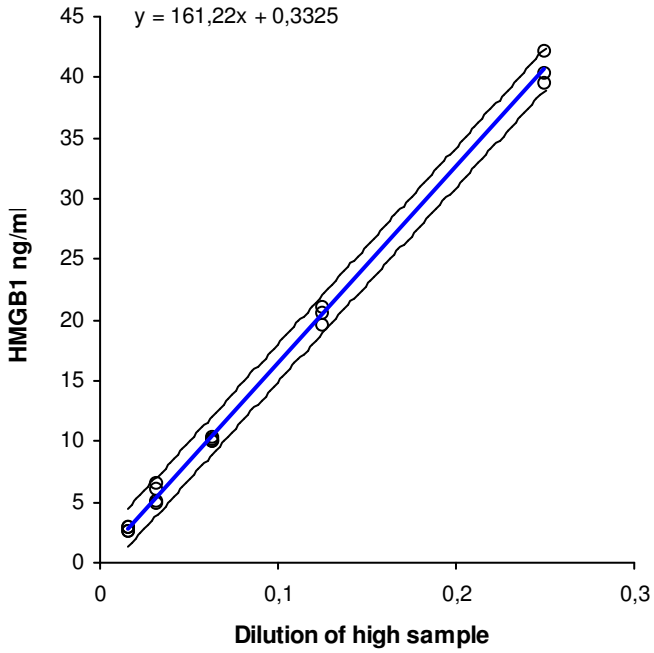
Traceability
No known international standard. Human HMGB1 has been purified by Yamada (see refs).

Trueness/Method comparison
No known reference method. Comparison with western blot a possibility, but not performed.

Measuring range
The minimum detection limit based on 4 determinations on a blank sample was 0,5 ng/ml (mean+5SD).
The CV% of a diluted sample with a concentration of 0,6 ng/ml was 34% (12 determinations) and 24 % when using duplicate measurements (n=6).
Detection limit as stated by the manufacturer: <1 ng/ml.
The assay was linear throughout the calibration range (see below).
Measuring range: 0,6 – 93,8 ng/ml.
The range can be broadened by dilution of high samples.

Specificity
Hemolysis, bilirubinemia, lipemia: Not evaluated
EDTA, Citrate, Heparin: Not evaluated
Other: No cross reactivity to HMGB2 (information supplied by manufacturer).

Linearity
Dilution of high sample according to NCCLS-EP6-P with 3 or 4 determinations at each level. No outliers removed.



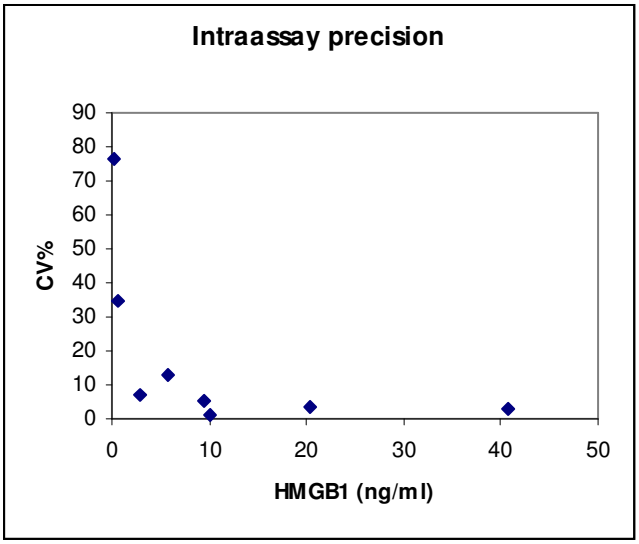
Good linearity throughout the calibration range

Precision

Intra-assay variation.

14 samples placed at various positions over the plate. Mean level 9.5 ng/ml, CV% = 5.0 (in duplicate measurements CV% = 3.6, n=7).

Precision varies through the measuring range (pooled data from precision experiments, linearity experiments, and detection level experiments):



The variation was less than 5% for samples above 10 ng/ml and around 10 % for samples 2-5 ng/ml.

Interassay variation.
The inter-assay variation determined from control-samples was 9.9 % at a level of 24.6 ng/ml (n=3, duplicates). (to be continuously expanded).

Robustness
Will be continuously evaluated

Pre-analytical factors
Not evaluated.

Practicability
Will be continuously evaluated

Budget of uncertainty

Pre-analytical:	5 %	(estimated)
Calibrator uncertainty	5 %	(estimated)
Analytical	9.9 %	
Total	12,2 %	

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XVII.2 Assay characteristics: sCD163, PCT, LBP, IL-6, IL-10

Variable	Analytical sensitivity	Precision		Calibration range	Recovery	Method
		Intraassay CV	Interassay CV			
sCD163	0.00625mg/l	3.6%	4.8%	6-200 ng/ml	92.5-115%	in-house ELISA
PCT	0.02ng/ml	2-3%	2-3%	0.02-5000 ng/ml		Kryptor PCT®
LBP	0.2 µg/ml	3.1-5.8%	3.6-10.6%	0.2-200 µg/ml	94-112%	Immulite-1000 ® DPC
IL-6	2 pg/ml	3.5-6.2%	5.1-7.5%	2-1000 pg/ml	85-104%	Immulite-1000 ® DPC
IL-10	1 pg/ml	2.8-3.4%	4.2-9.9%	1-1000 pg/ml	94-110%	Immulite-1000 ® DPC

References

sCD163
Moller HJ et al. Characterization of an enzyme-linked immunosorbant assay for soluble CD163. Scand J Clin Lab Invest 2002, 62: 293-299

Moller HJ et al. Biological variation of soluble CD163. Scand J Clin Lab Invest 2003, 63: 15-21

PCT
http://www.procalcitonin.com/default.aspx?tree=_4_0_1&key=kryptor3

LBP
http://diagnostics.siemens.com/siemens/en_GLOBAL/gg_diag_FBAs/files/package_inserts/immulite/Inflammation_Markers/lklb-9_int.pdf

IL6
http://diagnostics.siemens.com/siemens/en_GLOBAL/gg_diag_FBAs/files/package_inserts/immulite/Inflammation_Markers/lk6p-14_int.pdf

IL10
http://diagnostics.siemens.com/siemens/en_GLOBAL/gg_diag_FBAs/files/package_inserts/immulite/Inflammation_Markers/lkxp-9_int.pdf

XVII.3 Database

A database was established in Access.

The following variables were registered:

Patient data & inclusion:

1. Case record file
2. Study group
3. Date of admission
4. Birthday
5. Age
6. Sex
7. Inclusion criteria
8. Exclusion criteria
9. Date of informed consent
10. Weight
11. Height
12. Glasgow coma scale
13. Work
14. Myocardial infarction
15. Hearth failure
16. Peripheral vascular disease
17. Cerebrovascular disease
18. Dementia
19. Chronic lung disease
20. Connective tissue disease
21. Peptic ulcer disease
22. Mild liver disease
23. Diabetes
24. Hemiplegia
25. Moderate/severe kidney disease
26. Diabetes with complications
27. Malignant tumour
28. leukaemia
29. lymphoma
30. moderate/severe liver disease
31. metastatic solid tumour

32. AIDS
33. Charlson Index of co-morbidity
34. Other diseases
35. Other risk factors for sepsis
36. Medication at admission
37. Immunosuppressive treatment
38. Number of days with symptoms related to the actual admission
39. Tobacco consumption
40. Alcohol consumption
41. Usage of narcotics
42. Tentative infectious diagnosis at admission
43. Which tentative infectious focus
44. Other infectious focus

Antibiotics prior and on admission

1. Antibiotics prior to admission
2. Which antibiotics
3. Antibiotics at the time of admission

Microbiology

1. Bacteremia
2. Sputum cultures
3. Urine cultures
4. Other cultures
5. Viral pathogens
6. Fungal pathogens
7. Other pathogens

Radiology and Nuclear medicine

1. Chest X-ray
2. CT thorax
3. UL-Sound abdomen
4. CT abdomen
5. Other radiology
6. Nuclear medicine modalities

Routine biochemistry

1. potassium
2. ALAT

3. bilirubin
4. urea
5. creatinine
6. PP%
7. Natrium
8. hemoglobin
9. hematocrit
10. platelets
11. PCO2
12. Standard bicarbonate
13. pH
14. PO2

SOFA

1. Respiratory SOFA score
2. Coagulation SOFA score
3. Liver SOFA score
4. Cardiovascular SOFA score
5. Central nervous system SOFA score
6. Kidney SOFA score
7. Total SOFA score

Investigator classification, diagnoses, intensive care therapy, semi-intensive care therapy, survival

1. SIRS criteria
2. Maximal no. SIRS criteria
3. Verified infection
4. Severity of infection
5. Action diagnoses
6. Other infection
7. Intensive care therapy
8. Days in ICU
9. Date of discharge
10. Length of admission
11. Diagnosis of discharge
12. Survival
13. Mors date

XVII.4 Sepsis & severity definitions

Sepsis:

SIRS + Infection

Severe sepsis:

Sepsis combined with one or more of the following:

1. Glasgow coma scale of less than or equal to 14
2. PaO2 of less than or equal to 9.75 kPa
3. Oxygen saturation of less than or equal to 92%
4. PaO2/FiO2 of less than or equal to 250
5. Systolic blood pressure of less than or equal to 90 mm Hg
6. Systolic blood pressure decrease of more than or equal to 40 mm Hg
7. pH of less than or equal to 7.3
8. Lactate of more than or equal to 2.5 mmol/l
9. Creatinine of more than or equal to 177 µmol/l
10. 100% increase of creatinine in patients with known kidney disease
11. Oliguria of less than or equal to 30 ml/h in more than 3 h or less than or equal to 0.7 l/24h
12. Prothrombin time of less than or equal to 0.6 (reference 0.7-1.3)
13. Platelets of less than or equal to 100X10E9/l
14. Bilirubin of more than or equal to 43 µmol/l
15. Paralytic ileus

Septic shock:

Hypotension persisting despite fluid resuscitation for at least one hour

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XVII.5 Gold-standard definition on infection in the Ph.D study

The presence of infection was defined by at least one of the following:

1. Cultures/microscopy of a relevant pathogen from a clinical focus:
    - Cerebrospinal fluid
    - Blood cultures
    - Sputum
    - Urine (with symptoms)
    - Abscess
    - Other
2. Positive urine dip test with symptoms
3. Chest X-ray verified pneumonia
4. Other imaging technique/nuclear medicine modality
5. Obvious clinical infection (i.e. erysipelas, wound infection)
6. Identification of a relevant pathogen by antigen/antibody detection

XVII.6 Charlson Index of Comorbidity

Weighting of each disease between 1 and 6 points:

Weight: 1 point

1. Myocardial infarction
2. Congestive heart failure
3. Peripheral vascular disease
4. Cerebrovascular disease
5. Dementia
6. Chronic pulmonary disease
7. Connective tissue disease
8. Peptic ulcer disease
9. Mild liver disease
10. Diabetes

Weight: 2 points

1. Moderate/severe kidney disease
2. Chronic dialysis
3. Diabetes with end organ failure
4. Any malignant tumour
5. Leukaemia
6. Lymphoma

Weight: 3 points

1. Moderate/severe liver disease

Weight: 6 points

1. Metastatic solid tumour
2. AIDS

Charlson ME et al. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. J Chronic Dis 1987; 40: 373-383

XVII.7 Sequential Organ Failure Assessment (SOFA) score

Organ system	Variable	0	1	2	3	4
Respiratory	PaO2/FiO2 or O2 saturation[a]	≥400 or ≥98%	<400 or 97-90%	<300 or 89-80%	<200 with respiratory support or <79	<100 with respiratory support
Coagulation	Platelets (10E9/l)	≥150	<150	<100	<50	<20
Liver	Bilirubin (µmol/l)	<20	20 to 32	33 to 101	102 to 204	>204
Cardiovascular	Hypotension	≤70	≤70 only fluid	≤70 *	≤70 **	≤70 ***
Brain	Glasgow Coma Scale	15	13 & 14	10 to 12	6 to 9	<6
Kidney	Creatinine (µmol/l)	<110	110 to 170	171 to 299	300 to 440	>440

[a] Modification (inclusion of Oxygen saturation as variable for respiratory failure)
*Dopamine ≤ 5 µg/kg min for at least 1 h or dobutamine (any dose)
**Dopamine > 5 µg/kg min or epinephrine ≤ 0.1 µg/kg min or norepinephrine ≤ 0.1 µg/kg min
***Dopamine > 15 µg/kg min or epinephrine > 0.1 µg/kg min or norepinephrine > 0.1 µg/kg min

Reference

Vincent JL 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. Int Car Med 1996; 22: 707-710

XVII.8 Blood sampling procedures

Guidelines for the laboratory technicians sampling patients at the medical wards of Odense University Hospital:

Projekt 01-31 Procalcitonin

Sampling:

EDTA Vacutainer (purple stopper) n=2

Serum Vacutainer (red stopper) n=1

Sample preparation:

Within 1.5 h after sampling

1 EDTA Vacutainer (purple stopper) and 1 Serum Vacutainer (red stopper): Centrifuged with routine programme 3.

Serum was transferred to 2 NUNC tubes 1.8 ml and labelled "Serum"

Plasma was transferred to 2 NUNC tubes 1.8 ml and labelled "Plasma"

Whole blood from the last EDTA Vacutainer was transferred to 2 NUNC tubes 1.8 ml

Freezing:

The samples were frozen at minus 80º C in a box labelled "01-31"

Laboratory documentation file:

These files were stored in the research laboratory of the Dept. Clinical Biochemistry, Odense University Hospital

Other sampling aspects:

On a weekly basis samples were transferred from the minus 80º C freezers in the Dept. Clinical Biochemistry, Odense University Hospital, to the minus 80º C Research freezers in our Research Unit.