Diplomarbeit

Clinical evaluation of automated multiplexed combined inflammation biomarker tests for prognosis and diagnosis in patients with systemic inflammatory response syndrome

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Graz, 1.April 2014 Matthias Reichsöllner

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Zusammenfassung

Evaluierung multipler automatisierter Entzündungsparameter für die Prognose und Diagnose von Patienten mit systemisch-inflammatorischem Response-Syndrom

Hintergrund: Sepsis, definiert als systemisches inflammatorisches Response-Syndrom (SIRS) ausgelöst durch eine Infektion, ist mit erheblicher Morbidität und Mortalität verbunden. Eine frühzeitige Diagnose ist für eine zielgerichtete anti-infektive Therapie sowie die Verbesserung der Prognose entscheidend. Bislang konnten einzelne Laborparameter die zeitaufwändige Blutkultur als Diagnostikum nicht ersetzen, sondern nur als Zusatzinformation der Krankheitseinstufung dienen. Daher ist der Ansatz dieser Studie bewährte sowie neue Laborparameter auf ihr Potential hinsichtlich der Diagnose und Prognose einer Bakteriämie/Fungämie bei Patienten mit SIRS zu untersuchen.

Methoden: 159 Patienten aus der Notaufnahme mit SIRS (Blutkulturen: 49 negativ, 49 *Staphylococcus aureus*, 51 *Escherichia Coli*, 10 *Candida* spp.), wovon 27 innerhalb von 30 Tagen nach Aufnahme verstarben, wurden in diese Studie eingeschlossen. Neben den Routine-Laborparametern CRP, PCT, suPAR, IL-6 und Kreatinin wurden IL-8, SAA, Cystatin C, NGAL, IL-10, Fibronectin, Thrombomodulin, Biotin, Substanz P aus eingefrorenem Plasma des Kollektivs quantitativ mittels Anagnostics Inflammations-Monitoring (kompetitive- und Sandwich-Immunoassays; Anagnostics Bioanalysis GmbH) bestimmt und statistisch mit Hilfe von receiver operating characteristics (ROC) Kurven analysiert.

Ergebnisse: Während PCT (p=0,001), suPAR (p=0,001), Biotin (p=0,004), IL-8 (p=0,009) und NGAL (p=0,046) bei Patienten mit Bakteriämie/Fungämie signifikant erhöht waren, zeigte Fibronectin (p=0,020) signifikant niedrigere Werte. In der Bakteriämie/Fungämie-Vorhersage zeigten sich die höchsten area under the curve (AUC) Werte für PCT mit 0,694 (62% Sensitivität, 67% Spezifität bei einem cut-off von 0,6 ng/mL), gefolgt von suPAR (0,660), Biotin (0,646) und IL-8 (0,625). Die Prognose der 30-Tage Mortalität ergab für suPAR (0,676), Biotin (0,663), IL-8 (0,637) und PCT (0,630) die größten AUC Werte.

Conclusio: PCT, suPAR, Biotin und IL-8 waren die vielversprechendsten Marker für die Vorhersage einer positiven Blutkultur sowie von 30-Tage Mortalität bei SIRS Patienten. Insgesamt war jedoch keiner der Biomarker zuverlässiger Prädiktor von Bakteriämie/Fungämie in Notaufnahme-Patienten mit SIRS.

Abstract

Clinical evaluation of automated multiplexed combined inflammation biomarker tests for prognosis and diagnosis in patients with systemic inflammatory response syndrome

Background: Sepsis, defined as systemic inflammatory response syndrome (SIRS) due to infection is associated with substantial morbidity and mortality. Early diagnosis for an appropriate treatment proves to be vital in the quest to ameliorate its prognosis. While so far no biomarker was able to substitute time-consuming blood cultures, some are used for additional assessment. In this study we investigated the potential of established as well as new biomarkers regarding diagnosis and prognosis of bacteremia/fungemia in patients with SIRS.

Methods: 159 patients presenting with SIRS at the emergency department (blood cultures: 49 negative, 49 *Staphylococcus aureus*, 51 *Escherichia Coli*, 10 *Candida* spp.), 27 of whom died within 30 days after admission, were included in this study. Apart from the routinely tested CRP, PCT, suPAR, IL-6 and creatinine, frozen plasma from the patient population was used to determine levels of IL-8, SAA, cystatin C, NGAL, IL-10, fibronectin, thrombomodulin, biotin and substance P with the help of Anagnostics' inflammation-monitoring (competitive- and sandwich-immunoassays; Anagnostics Bioanalysis GmbH). The data was analysed statistically including receiver operating characteristics (ROC) curve analysis.

Results: PCT (p=0,001), suPAR (p=0,001), Biotin (p=0,004), IL-8 (p=0,009) and NGAL (p=0,046) were elevated significantly in patients with bacteremia/fungemia, while fibronectin levels were diminished (p=0,020). The highest area under the curve (AUC) value in the prognosis of bacteremia/fungemia exhibited PCT with 0,694 (sensitivity 62% and specificity 67% for a cut-off at 0,6 ng/mL), followed by suPAR (0,660), Biotin (0,646) and IL-8 (0,625). The best performance in the prognosis of 30-days mortality was accomplished by suPAR (AUC 0,676), Biotin (AUC 0,663), IL-8 (AUC 0,637) and PCT (AUC 0,630).

Conclusion: PCT, suPAR, biotin and IL-8 were the most promising biomarkers for the prediction of positive blood cultures as well as 30-days mortality in patients with SIRS. Overall, however, none of the investigated biomarkers appeared as reliable predictor of bacteremia/fungemia in patients from the emergency department presenting with SIRS.

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Glossary and Abbreviations

AKI	acute kidney injury
A-SAA	acute-phase serum amyloid A
ATP	adenosine triphosphate
AUC	area under the curve
C3b	complement component 3b
CARS	compensatory anti-inflammatory response syndrome
CI	confidence interval
CKD	chronic kidney disease
CRP	C-reactive protein
C-SAA	constitutive serum amyloid A
CSIF	cytokine synthesis inhibitory factor
DIC	disseminated intravascular coagulation
eGFR	estimated glomerular filtration rate
GFR	glomerular filtration rate
HIV	human immunodeficiency virus
IL-1	interleukin-1
IQR	interquartile range
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MDRD	Modification of Diet in Renal Disease
MODS	multiorgan dysfunction syndrome
mRNA	messenger ribonucleic acid
MRSA	methicillin resistant Staphylococcus aureus
mv	mean value
n	valid numbers counted
NGAL	neutrophil gelatinase-associated lipocalin
OR	odds ratio
PAF	platelete activating factor
PCT	procalcitonin
ROC	receiver operating characteristics
r_s	Spearman’s rho / rank correlation coefficient

SAA	serum amyloid A
SIRS	systemic inflammatory response syndrome
std	standard deviation
suPAR	soluble urokinase-type plasminogen activator receptor
THBD	thrombomodulin
TLR	toll-like receptor
TNF-α	tumor necrosis factor α
uPA	urokinase-type plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
WBC	white blood cell count

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1 Introduction and Background

In 1992 the ACCP/SCCM consensus conference defined Sepsis as a number of clinical conditions easy to measure, called Systemic Inflammatory Response Syndrome (SIRS) combined with a confirmed infectious process (1). Since then the definition has remained unchanged, however, a lot of scientific effort has been put into the challenge to find biomarkers for a quicker and more accurate diagnosis of infection in order to provide earlier and more specific anti-infective treatment as time is one of the most important factors in the quest to reduce mortality and morbidity of sepsis (2,3).

1.1 SIRS and Sepsis

1.1.1 Definition of SIRS

In order to confirm SIRS as an inflammatory state of the entire organism – regardless of the location or specific insult which caused a systemic inflammatory response, two or more of the following conditions must be found in a patient (1):

- Temperature over 38°C or under 36°C [rectal, intravascular or intravesical measurement(4)]
- Heart rate over 90 beats per minute
- Respiratory rate over 20 breaths per minute or p_aCO_2 under 32 mmHg
- White blood cell count (WBC) over 12000/mm3 or under 4000/mm3 or over 10% immature (band) Neutrophiles

Figure 1 shows, that SIRS does not necessarily come from an infection and it does not always lead to sepsis. Therefore the consensus conference in 1992 proposed the phrase systemic inflammatory response syndrome which indicates an inflammatory process with various possible causes like trauma, burns, pancreatitis or – in which case we speak of Sepsis – infection (1).

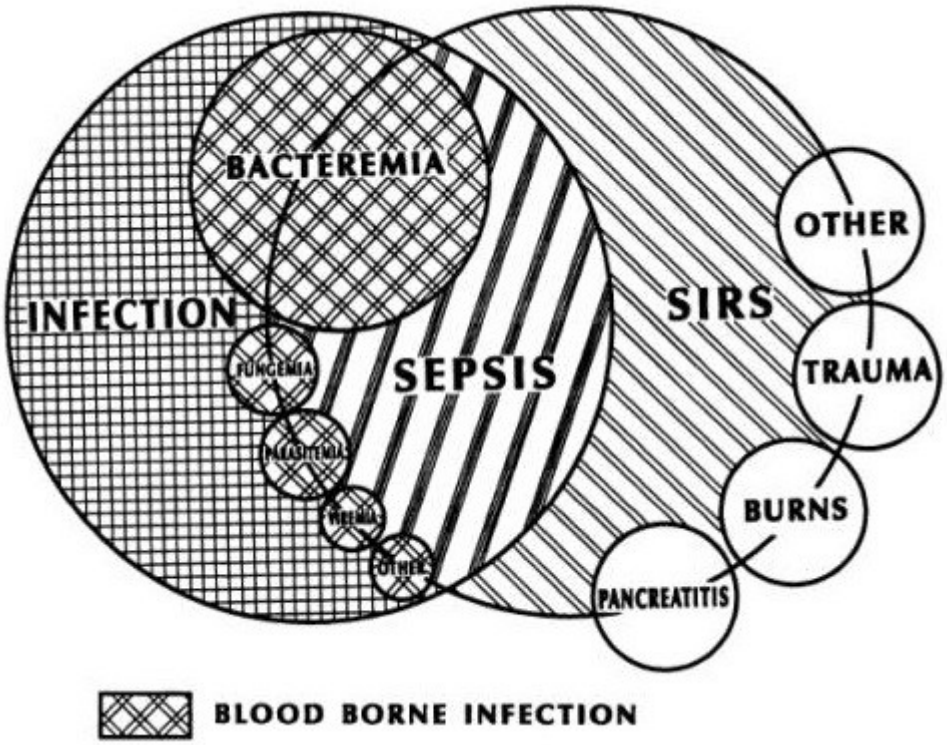

Figure 1: The connection between infection, SIRS and sepsis [adapted from (1)]

1.1.2 Sepsis, Severe Sepsis and Septic Shock

As mentioned above, sepsis is the inflammatory response to an infection – confirmed or suspected. The causative pathogens that make SIRS to a septic infection are gram-negative and gram-positive bacteria as well as fungi. Further distinctions of sepsis are made by its severity: s*evere sepsis* means sepsis with organ dysfunction, hypoperfusion and hypotension whereas patients in *septic shock* suffer from sepsis-induced hypotension in spite of adequate fluid replacement or are in the need of vasopressors to maintain a systolic arterial pressure above 90 mmHg. (1,4)

Criteria for SIRS, sepsis, severe sepsis and septic shock are summarized in the following Table 1.

I systemic inflammatory response syndrome SIRS (at least 2 applicable)

- **temperature > 38°C or < 36°C [rectal, intravascular or intravesical measurement(4)]**
- **heart rate > 90/min**
- **respiratory rate > 20/minute or p_aCO_2 < 32 mmHg**
- **WBC > 12,000/mm3 or < 4,000/mm3**
  or immature (band) neutrophils > 10%

II sepsis

- **SIRS criteria fulfilled**
- **immanent infection**

III severe sepsis

- **sepsis**
- **organdysfunction (at least 1 applicable)**
  - **encephalopathy: constrained vigilance, disorientation, delirium**
  - **thrombocytopenia: thrombocytes < 100.000/mm3 or loss > 30% in last 24 hours (loss due to haemorrhage must be ruled out)**
  - **arterial hypoxemia: p_aO_2 ≤ 10kPa (≤ 75 mmHg) or p_aO_2/FiO_2-ratio ≤ 33 kPa (≤ 250 mmHg) under application of oxygen (existing causative heart or lung diseases must be ruled out)**
  - **renal dysfunction: diuresis ≤ 0,5 ml/kg/h over 2 hours despite adequate fluid resuscitation or serum-creatinine more than doubled**
  - **metabolic acidosis: base excess ≤ -5 mmol/L or lactate rise more than 1,5 times the usual range**

IV septic shock

- **sepsis**
- **at least one hour of: systolic arterial pressure ≤ 90 mmHg or mean arterial pressure ≤ 65 mmHg or arterial pressure can only be held above these limits with vasopressors (hypotension despite adequate fluid resuscitation)**

Table 1: Criteria for SIRS, sepsis, severe sepsis and septic shock [adapted from (1,4)].

1.1.3 Immunopathogenesis – from Infection to Sepsis

1.1.3.1 Contact with Microbial Antigens

In the beginning of an infection the inflammatory response starts when the immune system gets in contact with microbial macromolecules, so called endotoxins like lipopolysaccharid (LPS), an essential component of the wall of gram-negative bacteria (Figure 2). Apart from LPS various other microbial molecules such as peptidoglycan (gram-positive and –negative), lipoteichoic acid (LTA) (gram-positive) and other structures of bacteria, fungi and viruses can trigger an immune response. A healthy individual's immune system should be able to react instantly to maintain homeostasis of the organism. (5,6)

Some of the key molecules in this process are lipopolysaccharide-binding protein (LBP), the endotoxine receptor mCD14 and the toll-like receptors (TLR) 2 and 4 on the surface of many immunocompetent cells. The purpose of LBP, an acute-phase plasma protein is – as the name indicates – to bind LPS. The membranous mCD14 receptor on the other hand can bind numerous microbial molecules. CD14 can also be found circulating as soluble sCD14. The TLR's role is not only the recognition of pathogen associated pattern but also the downstream signal transduction after contact with microbial antigens. TLR-4 is known to activate a number of intracellular kinases which after a series of other reactions leads over nucleic factor kappa B (NF-kB) pathway to the release of pro-inflammatory cytokines, platelet activating factor (PAF), tissue factor and other mediators.(5,6)

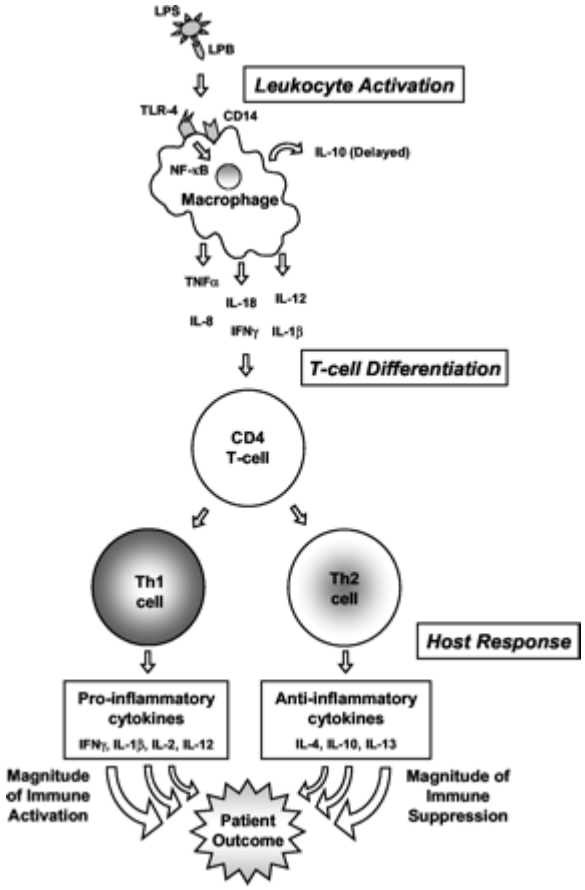

Figure 2: Schematic illustration of host response after contact with LPS [adapted from (6)].

1.1.3.2 Cytokines as Mediators

Even before the above described pathway was discovered, by injecting pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) into laboratory animals it was shown that those cytokines provoked the same sepsis-like symptoms that can be observed when injecting LPS. By being able to neutralise the cytokines with antibodies which reduced the symptoms distinctly, cytokines where identified as essential mediators in inflammatory processes. The pathogenesis of sepsis, however, is not only characterised by pro-inflammatory response (Figure 2 and Figure 3): after an individually varying delay of a few days the compensatory anti-inflammatory response syndrome (CARS) sets in with the release of anti-inflammatory cytokines like IL-4 and IL-10 which then are also responsible for the decrease of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 or IL-8. This late CARS-phase of sepsis is characterised by an anergy of the immune system (lack of reaction by the body's defence mechanisms

to antigens in vivo (7)) – supposedly by apoptosis of many immunocompetent cells – which is linked to a high mortality rate despite extensive treatment.(5,6)

Thus the immune system can be seen as two edged sword with a pro- and anti-inflammatory part that cannot only eradicate bacteria effectively but might also damage the integrity of the body (8). Attempts to simply block either the pro- or anti-inflammatory response have proven ineffective and treatment of sepsis may not be as easy as aiming at pro- or anti-inflammatory agents alone but to maintain their homeostasis and try to identify the causative pathogens for an effective eradication (8-10).

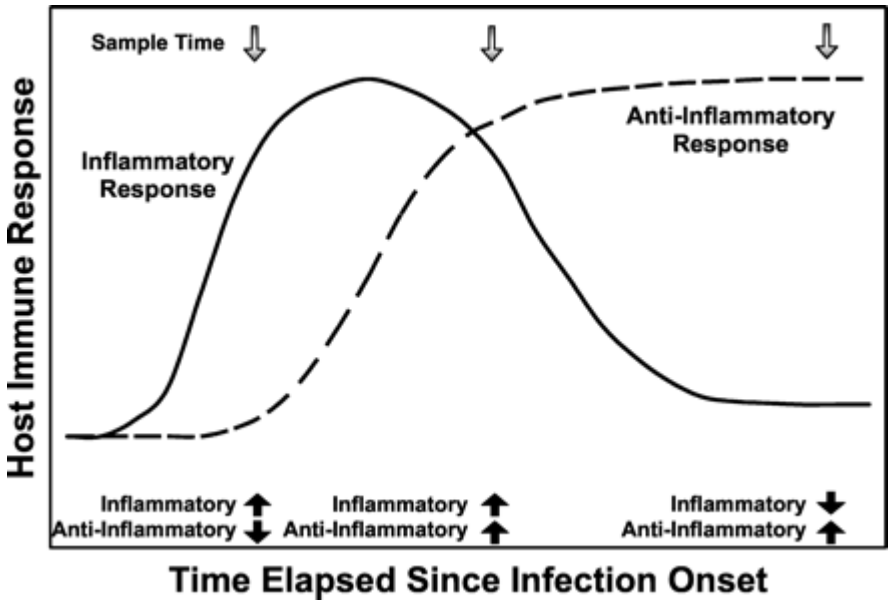

Figure 3: Simplified pro- and anti-inflammatory host response to infection [adapted from(6)].

1.1.3.3 Sepsis – more than Pro- and Anti-Inflammatory Response

During sepsis patients frequently suffer from disseminated intravascular coagulation (DIC) caused by a consumption of platelets and clotting factors based on various alterations within the coagulation system and regulatory mechanisms (11). Despite the fact that the interaction between clotting system, platelets, white blood cells and endothelium has been documented, the cause of coagulopathy during sepsis seems to be multifactorial (12).

Furthermore cellular dysfunction can strike both ways: a delayed removal of redundant and/or hyperactive cells - in some cases neutrophils which can add to

the toxicity - or on the other hand a too early and excessive removal/apoptosis/necrosis of cells that are needed - i.e. lymphocytes (12). Moreover it has been demonstrated that other cells like dendritic cells, macrophages/monocytes, mucosal epithelial cells and others increasingly undergo apoptosis and stop their normal function in the presence of sepsis (13).
Remick, D.G. (12) recently not only pointed out the numerous immunopathologic alterations in septic patients, but also referred to numerous controversies and questions in the topic of SIRS and sepsis which remain unanswered.

1.1.3.4 Non-infectious Causes of SIRS

In addition to the infectious systemic inflammatory response caused by various pathogens as described in the previous chapters, SIRS can also be provoked by chemical, traumatic or other nonspecific insults to the body. Regardless of aetiology, the pathophysiologic cascades show minor differences in the inflammatory response apart from the missing microbial surface molecules in non-infectious patients which trigger immune response in infectious patients. Nonetheless cytokines are released following an insult with the intention to unleash an inflammatory response and encourage wound repair and recruitment of the mononuclear phagocyte system. To support local response small amounts of cytokines are released into the circulation. If homeostasis cannot be restored, a systemic inflammatory response is inevitable following similar patterns as in infectious cases.(14)
However, the discrimination between infectious and non-infectious insults is not always easy, given that infection often occurs in the process. Literature suggests that sepsis is even more common as a complication after treatment of trauma, cancer or other major surgeries (15).

1.1.4 Epidemiology and Pathogens

A national prospective multicenter study in Germany extrapolated that in 2003 79.000 (116 out of 100.000) inhabitants suffered from sepsis and 75.000 (110 out of 100.000) developed severe sepsis. Hence sepsis represents the seventh frequent discharge diagnosis in German hospitals and when it comes to causes of

death, sepsis is even ranked third. Despite 68% of patients with sepsis being older than 60 years, the mortality rate of those younger than 60 was as high as 46% compared to 60% mortality rate in patients older than 60. These figures show that sepsis is not only a disease of old and multimorbid but also younger patients.(16,17)

An American epidemiological study analysing hospital records between 1979 and 2000 unveiled an average annual increase in sepsis of 8,7% or in other figures: in the 22-year period of the study, the incidence of sepsis rose from 82,7 to 240,4 cases per 100.000. Specific organisms were detected in 51% of all discharge records, the most common being gram-positive bacteria with 52,1% followed by gram-negative bacteria accounting for 37,6% of all microbial pathogens. Fungi accounted for 4,6%, anaerobic bacteria 1% and polymicrobial organisms were found in 4,7%.(18)

The clearly noticeable increase in gram-positive cases at a mean annual rate of 26,3% found in the epidemiological study during 1979 and 2000 (18) might be explained by a rising number of patients under chemotherapy, immunosuppression or catheterisation (8), and might as well sound alarming by looking at some other surveillance numbers of isolated methicillin resistant *Staphylococcus aureus* (MRSA) ranging from 29 to 45% of overall collected clinical specimens (6,19,20).

1.2 The PIRO-concept

The acronym PIRO stands for Predisposition Infection Response Organ dysfunction and acknowledges the presumed need for individualisation in the diagnosis and treatment of SIRS and sepsis patients (6). Although the terms sepsis, severe sepsis and septic shock tell us something about the severity of the condition, these definitions are not precise enough to characterise them. PIRO is an attempt to stage septic patients just like the well-known TNM staging system in oncology where the extent of the primary tumour (T), metastases in regional lymph nodes (N) and distant metastases (M) not only influences or determines the steps of treatment but also correlates with prognosis and survival (6,21).

Predisposition indicates the influence of factors like genetic susceptibility, resistance to antimicrobials or premorbid health status of the patient whereas *Infection* describes its significant impact of type, location and extent on prognosis. *Response* refers to the host response measuring biomarker and mediators as well as other symptoms to help determine a therapeutic stratification. Finally, *Organ dysfunction* adds to the determinants of sepsis prognosis.(6,21,22)
Nevertheless, timely treatment is crucial for a positive outcome which depends on prompt and accurate diagnosis PIRO fails to meet this condition (6).

1.3 Diagnosis of Sepsis

For sepsis as a complex systemic inflammatory host response to an infection, there is no such thing like a single parameter that could determine the diagnosis. At present, sepsis, severe sepsis and septic shock are looked upon as a continuum of conditions, defined by a combination of vital and hemodynamic parameters as well as organ function or dysfunction.(4)
Depending on preceding anti-infective therapy, bacteremia can only be found in an average 30% of patients with severe sepsis or septic shock which underlines the difficulties of diagnosis (4,18,23-26). Further on, patients who are most likely to have an infection according to clinical criteria show negative microbial cultures in about 30% (27,28).

1.3.1 Blood Culture

Since the detection of bacteremia and fungemia is one of the most important tasks of clinical microbiology, especially when dealing with systemic inflammatory response syndrome and suspicious infectious processes, the microbiological culturing of blood – a usually sterile environment – has become absolutely essential. Clinically relevant microorganisms in blood cultures are signs of a failed host defence which is unable to contain an infection at its primary site.(29)
German sepsis guidelines (4) recommend obtaining blood cultures, if sepsis is suspected or one of the following conditions apply: fever, shivering attack, hypothermia, leukocytosis, deviation to the left of differential blood count, elevated serum levels of procalcitonin or C-reactive protein (CRP) and neutropenia. Most

beneficial of culturing is not only the detection of microorganisms, but also the ability to examine antibiotic resistance and therefore develop effective treatment. For further principles of taking blood cultures, see Table 2.

Withdrawal of blood cultures: always in advance of anti-infective treatment; in the end of therapeutic break or ahead of next dose, if anti-infective treatment already started
Aseptic procedure when withdrawing blood
Blood volume: 10 ml for each aerobic and anaerobic bottle
Spots of withdrawal: 2-4 pairs of aerobic and anaerobic blood cultures from different takings, if need be also from intravascular catheters
Important information: date, time and locus of withdrawal, underlying diseases, risk factors, suspected diagnosis, previous antimicrobial treatment
Transfer to microbiology: as fast as possible, in any case < 16 hours after withdrawal, interim storage only over night

Table 2: Principles for taking blood cultures [adapted from (4)].

Despite blood cultures being the gold standard for the diagnosis of infection, first results require in most cases at least 24 hours, even 33 hours at an average. In addition to slowness of this method, many patients suspected to have sepsis - meaning infection - show negative cultures due to small volume samples, transient bacteremia, and sepsis of non-bacterial origin or previous antibiotic treatment.(3)
One study pointed out that two blood cultures detected only 80% of blood stream infections but with more blood cultures taken, the sensitivity would increase: three detected 96% and four were needed to detect all blood stream infections (30).
On the other hand, positive results also need to meet with cautiousness, given the fact that they might be accounted for by contamination, especially when detecting coagulase-negative *staphylococci*, *Propionibacterium, Corynebacterium* or α-haemolytic *Streptococcus* species (31).
Considering the long timeframe until results are available, the low sensitivity as well as the vulnerable specificity (contamination) of this method, more rapid and reliable techniques are required (3).

1.3.2 Laboratory Biomarkers for Sepsis Diagnosis

A biological marker (biomarker) can be defined as an objective characteristic to measure and assess not only normal biological processes, but especially pathogenic processes, or pharmacologic responses to a therapeutic intervention. Therefore biomarkers are most helpful in the detection of diseases and the monitoring of health status.(32)

According to the current guidelines of the German sepsis-society (4), procalcitonin (PCT) is recommended to rule out severe sepsis, as a progression parameter and to back up the diagnosis of sepsis. Alongside PCT, especially CRP but also interleukin-6 (IL-6) are widely used biomarkers for sepsis diagnosis (6,33,34).
One major cause why hopes are pinned on biomarkers for sepsis diagnosis and improvement of the outcome is that they rise in the early hours of an inflammatory response - earlier than blood cultures could turn positive - some cytokines even within the first one to three hours like TNF-α or IL-6 (35).

1.3.2.1 Procalcitonin

PCT is the precursor of calcitonin, one of the regulatory hormones to keep a steady plasma calcium level. The polypeptide PCT consists of 116 amino acids and the Calc-1 gene on chromosome 11 regulates its synthesis. In healthy organisms calcitonin and consequently PCT are solely produced by the C-cells of the thyroid gland. As PCT is cleaved post-translationally and processed to the biologically active calcitonin, healthy individuals show very low to even undetectable serum levels of PCT (below 0.05 ng/mL). Neuroendocrine tumors like medullary thyroid cancer, small cell lung cancer or carcinoid tumors can increase the serum concentrations of PCT and calcitonin.(36)
The induction of Calc-1 gene, however, is not always restricted to the neuroendocrine C-cells but can be detected in extra-thyroidal tissue such as liver, kidney, pancreas, adipose and white blood cells, stimulated by microbial toxins or inflammatory mediators like several interleukins or TNF-α. Therefore, systemic inflammatory processes raise the production of PCT throughout the entire body. However, non-neuroendocrine cells lack the ability of PCT-modification to its

biologically active successor which makes PCT and not calcitonin to a valid sepsis biomarker.(36)

Although the pathophysiologic role of PCT in the immune response is still not entirely clear (37-39) it is the only marker currently recommended in the S2 guidelines for supplementary sepsis diagnosis (4). Serum concentrations of PCT lower than 0,5 ng/mL object to severe sepsis and septic shock whereas concentrations above 2,0 ng/mL blood culture almost prove their existence (4,40-43). Leukopenic patients on the other hand show lower PCT concentrations during sepsis and even less than 2,0 ng/mL during septic shock (44), which compromises its diagnostic potential. One randomized trial also showed that regular measurement of PCT and analysis of its progression during treatment could reveal possible reduction of anti-infective therapy by three to four days without apparent harm (45). If compared with blood culture results, PCT approaches high sensitivity but lacks specificity as well as the power to distinguish between bacterial and fungal sepsis (3). However, both gram-positive and gram-negative bacterial infections cause an increase of PCT serum levels that does not differ significantly (46). Additionally, some studies state, that PCT seems to increase after various inflammatory insults like hepatic dysfunction, trauma, anti-T-cell therapy, burns, heat stroke or fungal infection and therefore has no diagnostic use for differentiating the causes of inflammation which means it cannot tell the clinician whether he is dealing with sepsis or other non-infectious triggers for systemic inflammatory response syndrome (6,47).

1.3.2.2 C-reactive protein

In 1930 C-reactive protein was first detected in the course of studies of *Streptococcus pneumonia* infected patients. It was described as an acute phase protein that precipitated the “C” polysaccharide from the bacterial cell wall. Four decades later this polysaccharide was identified as phosphocholine, part of the teichoic acid of *S. pneumonia* and was only the first of numerous discovered ligands of CRP.(48)

Serum or plasma levels of CRP can increase thousand-fold due to an enhanced synthesis by hepatocytes as a response to acute inflammation in the body. The

gene of CRP is located on the short arm of chromosome 1 and its induction and CRP production is principally regulated by cytokines like IL-6 and IL-1β. Both these cytokines are involved in the expression of various other acute phase proteins; hence CRP stimulus is only part of multiplex liver gene expression during inflammatory conditions. At present no less than 40 plasma proteins are considered acute phase proteins comprising clotting proteins, complement factors, anti-proteases, and transport proteins. While those proteins of the acute phase response increase, the syntheses of others - like albumin - are restricted after an inflammatory stimulus. Apart from the liver, CRP synthesis has been discovered in monocytes, lymphocytes, neurons as well as atherosclerotic plaques but is not considered significantly enough to influence levels of CRP. Also regulating mechanisms at these sites remain unknown.(48)

Similar to other inflammation mediators, both pro- and anti-inflammatory effects are described, last of which being the induction of IL-1 receptor antagonist, enhancement of IL-10 release or suppression of interferon-γ. Alternatively many pro-inflammatory functions are described: not only aggregated but also CRP bound to ligands activates the complement system which acts as host-defence mechanism against germs; the interaction of CRP with immunoglobulin receptors engages the response of phagocytic cells; CRP also increases the release of IL-1, IL-6, IL-18 and TNF-α.(48)

Minor CRP elevations have historically been considered insignificant but recent research suggests that slightly elevated plasma levels between 3 – 10 µg/mL could be associated with a higher risk of cardiovascular disease, metabolic syndrome, and colon cancer and might also play a role in the pathogenesis of atherosclerosis. (48)

Admittedly research on CRP as biomarker for septicemia in patients with SIRS often appears to be inconsistent with some results claiming a high value while others do not favour CRP as a diagnostic tool for SIRS, respectively sepsis. Serum or plasma-concentrations of CRP in patients with sepsis (range from 12 to 159 mg/L) tend to overlap significantly with concentrations in SIRS patients (13 – 119 mg/L) and show low sensitivity and specificity.(2,6)

Although CRP is supposed to represent the severity of an inflammation process and increases significantly more in bacterial than in viral infections, it takes longer to be detectable than PCT after infection (49).

1.3.2.3 Interleukin-6

Inflammation as a process that combats infection or tissue injury is highly orchestrated by a vast number of factors involving interleukin-6, which is considered a pro- and anti-inflammatory cytokine and activator of the acute phase response. . Moreover, the transition from innate to acquired immune system seems to play a vital part in the resolution of an inflammatory episode including the precise regulation of leukocyte recruitment and removal. IL-6 is assumed to mediate and serve as an immunological switch between innate and acquired immune response (Figure 4). Therefore, IL-6 takes on a critical role in the management of acute inflammation, which is essential for a successful resolution of any inflammatory state.(50)

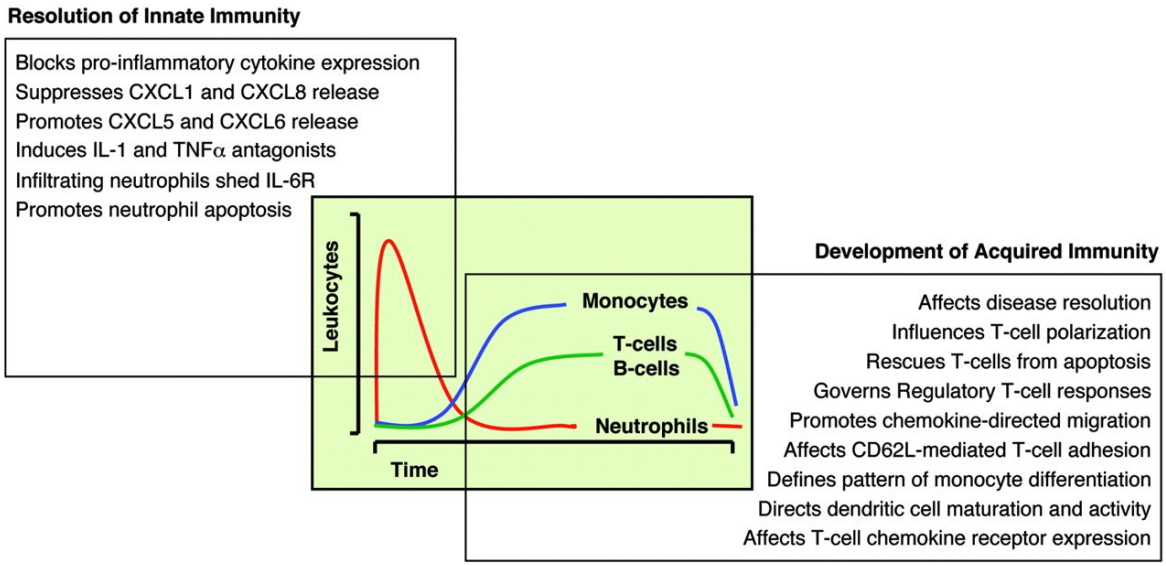

Figure 4: IL-6 plays a vital role in the regulation of innate and acquired immune response. The transition from a neutrophil- to mononuclear-dominated cell population in the middle section depicts the switch between innate to acquired immunity. A series of events on both wings, which are mediated by interleukin-6 are illustrated. [adapted from (50)].

A review on IL-6 in neonatal sepsis diagnosis showed its usefulness revealing serum levels from 47 to 1617 ng/L during sepsis compared to serum levels of

healthy neonates ranging from 2 to 42 ng/L. The sensitivity of IL-6 for neonatal sepsis diagnosis revealed by ROC analyses was also considered acceptable (6).

An assessment of IL-6 for the discrimination of SIRS and sepsis among 78 critically ill adult patients found an AUC value of 0,75 with a sensitivity and specificity of 67% and 72% (cut-off value 200 ng/L) (41).
Another study on 33 adult intensive care unit patients using IL-6 to distinguish between SIRS and sepsis found the best cut-off value at 68,5 ng/mL but with 0,515 showed an insufficient AUC value and disappointing sensitivity (51%) and specificity (53%) (51).
Accordingly, some reports demonstrate the increase of IL-6 in the early host response to infection with values ranging from 300 – 2700 ng/L in septic patients and above 100 ng/L in patients with SIRS, but some also indicate not enough significance to differentiate between sepsis and SIRS (6).

1.3.2.4 Soluble Urokinase-type Plasminogen Activator Receptor

Studies on the soluble form of the urokinase-type plasminogen activator receptor suPAR began in 1991 when its affinity towards urokinase-type plasminogen activator (uPA), also known as “urokinase”, was detected. uPA, a serin protease, is part of the plasminogen activating pathway which results in plasmin – a trigger of a proteolysis cascade aiming at thrombolysis or extracellular matrix degradation. Like its membrane bound predecessor, suPAR consists of three domains (DI, DII, DIII) and is detached from the membrane by cleavage of the GPI-anchor (Figure 5). (52)

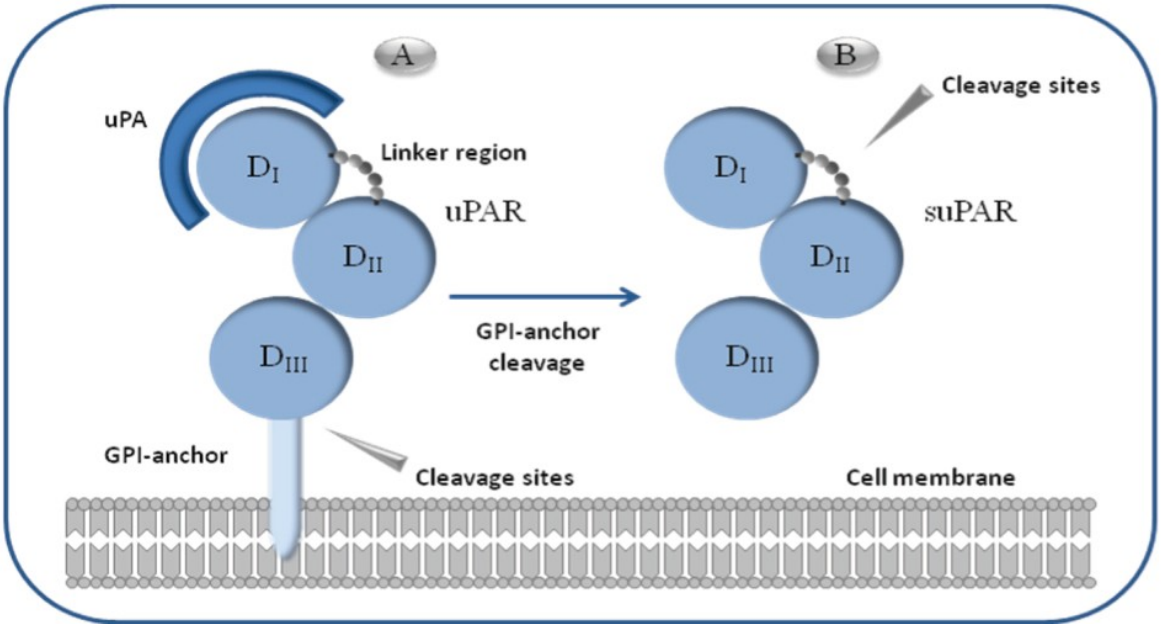

Figure 5: Relation of urokinase plasminogen activator (uPA), membrane bound- (uPAR) and soluble urokinase plasminogen activator receptor (suPAR) [adapted from (52)].

uPAR is expressed in immunocompetent cells like monocytes, activated T-lymphocytes, macrophages and also in endothelial cells, keratinocytes, fibroblasts, smooth muscle cells, megakaryocytes as well as some tumor cells. Body fluids carrying the soluble form are plasma, urine and cerebrospinal fluid. (52)
Cell migration is not only an essential part of the immune response after infection but also plays a vital role in invasive cancers and tissue remodelling after injuries. This migration via blood stream into certain tissues includes adhesion and chemotaxis with the uPAR/uPA system being directly involved in the process.(52)

Studies prove an increased expression of uPAR in inflammatory cells after cytokine stimulus as well as rising serum concentrations of suPAR in infectious and inflammatory conditions comprising infections such as HIV, malaria, rheumatoid arthritis, other viral infections and bacteremia with endotoxinemia.(53-57) Further investigations on patients with systemic inflammatory response syndrome found significantly higher suPAR serum levels in SIRS patients with infection, with elevations being most significant in cases of bacteremia (2,58,59). Hoenigl et al found an AUC value of 0,726 and a cut-off at 7,9 ng/mL with a sensitivity of 62 % and specificity of 77 % for suPAR in the quest to predict bacteremia in patients with SIRS (58).

SuPAR levels in healthy individuals' plasma seem to be stable throughout the day and circadian changes appear to be very limited (24 hours with 20 minutes intervals) (60).

1.3.2.5 Creatinine and Glomerular Filtration Rate

When a muscle is at rest, creatine kinase catalyses the reaction of ATP – a non-storable energy source – and creatine to creatinephosphate. When activated again, muscles reverse this reaction quickly to provide enough ATP when needed. A small, but consistent amount of the energy storage creatine (proportional to the muscular mass) is constantly metabolised to its excretion substance creatinine, which is the eliminated by the kidneys, particularly by glomerular filtration, but also depending on its plasma concentration by proximal tubular secretion. Besides, creatinine is not reabsorbed in the tubular system. In case of a deficient filtration of the kidney, creatinine blood levels rise. Decrease in creatinine serum levels is without further clinical relevance, however, the sensitivity of serum creatinine as biomarker for renal function is limited to the fact that it does not rise until glomerular filtration rate is down by 50% or more. Therefore, high levels of creatinine in patients of normal constitution indicate a restricted filtration rate, whereas low levels do not prove a proper renal function. As creatinine is the excretion substance of the muscular energy metabolism, interpretation of serum levels must always meet with considerations of individual muscular mass: a body builder with high creatinine serum levels may have a normal renal function, whereas the same levels in an elderly patient could mean significant renal damage. Consequently pre-renal increase can be found after bodybuilding, muscular trauma, burns and muscle dystrophy. (61)

Clinically most relevant causes of elevated serum creatinine are (list is not intended to be exhaustive) (61):

- Acute kidney injury (AKI)
- Chronic kidney disease (CKD)
- Congestive heart failure (pre-renal kidney injury due to hypoperfusion)
- Urinary tract obstructions
- Aminoglycosides, cytostatics

The glomerular filtration rate (GFR) depicts the flow rate of blood volume filtered through the kidneys' glomerula and is recorded in units of volume per time (typically mL/min). GFR is considered to be the best overall benchmark of renal function, most precisely determined by measurement of urinary/plasma clearance of an ideal (exogenous) filtration marker such as inulin - neither reabsorbed nor secreted after filtration in the renal glomerula. Due to complexity of measurement protocols and costs, alternatively but not being considered gold standard, endogenous creatinine clearance can be used instead, but still needs timed urine collection and blood sampling during collection period. Most commonly, however, GFR is estimated (eGFR) using the so called MDRD-equation (Modification of Diet in Renal Disease) which is based on serum creatinin levels.(62-64)

Since acute renal failure is a common complication of sepsis and by definition signifies severe sepsis, a significant decrease in GFR or increase of serum creatinine imply an ominous prognosis (65). Reports show higher mortality in patients with renal failure from sepsis than in non-septic cases (66).

1.4 Biomarkers measured with Multiplex Analysis System

1.4.1 Interleukin-8

The cytokine interleukin-8 (IL-8) is a member of the CXC family of chemokines and is produced by macrophages, endothelial and other cell types of the immune system and can be induced by inflammatory stimuli. Its primary function is to initiate chemotaxis, mainly in neutrophils (therefore also known as neutrophil chemotactic factor), causing immunocompetent cells to migrate towards the site of inflammation/infection and induce phagocytosis once these cells have arrived but is now also known to have tumorigenic and proangiogenic properties.(67,68)

IL-8 as diagnostic tool has mainly been investigated among new-born infants. One study that evaluated the diagnostic accuracy of IL-8 whole blood levels to predict positive blood cultures in new-borns found a sensitivity of 83% and specificity of 67% at a cut-off value of 1000 pg/mL (69). Another report among 40 neonates with

suspected infection revealed significantly higher levels of IL-8 in neonates with positive blood cultures compared to the non-infected group with a sensitivity and specificity of 62% and 96% (70). A third study regarding neonatal sepsis again confirmed significantly higher levels of IL-8 in infected than in healthy neonates with a AUC value of 0,68 (71).

1.4.2 Serum Amyloid A

Originally serum amyloid A comprised only one precursor of amyloid A, a protein which circulates in the blood stream and forms secondary amyloid plaques in the process of chronic inflammatory diseases that are deposited in organs. As of now serum amyloid A denotes a family of apolipoproteins attached to high-density lipoprotein, comprising two main groups differentiated by the response to inflammation: Acute-phase serum amyloid A proteins (A-SAA) and constitutive serum amyloid A (C-SAA) which, as the name implies, is expressed constitutively in the liver. The A-SAA fraction of the family has a crucial part in the acute-phase response of an inflammation and its increase is furthermore associated with chronic inflammatory diseases. On the other hand C-SAA shows only minimal elevation during acute-phase response. While the liver is considered to be the major site of production for both C-SAA and A-SAA, studies suggest additional extrahepatic synthesis of most serum amyloid A family members.(72,73)

After pro-inflammatory stimuli, mRNA of A-SAA is induced massively due to cytokines like interleukin-1 and interleukin-6, which have synergistic effects, but also due to stimulation by glucocorticoids (Figure 6). Various transcription factors that play a part in the induction of SAA have been identified.(72)
Even though the role of SAA in the host defence is not entirely understood, the apolipoprotein family might gain clinical relevance to assess inflammatory states (72). Although SAA still requires further investigations as a biomarker for sepsis diagnosis, it has already been analysed among septic neonates and is considered as a potentially promising tool for sepsis diagnosis (74).

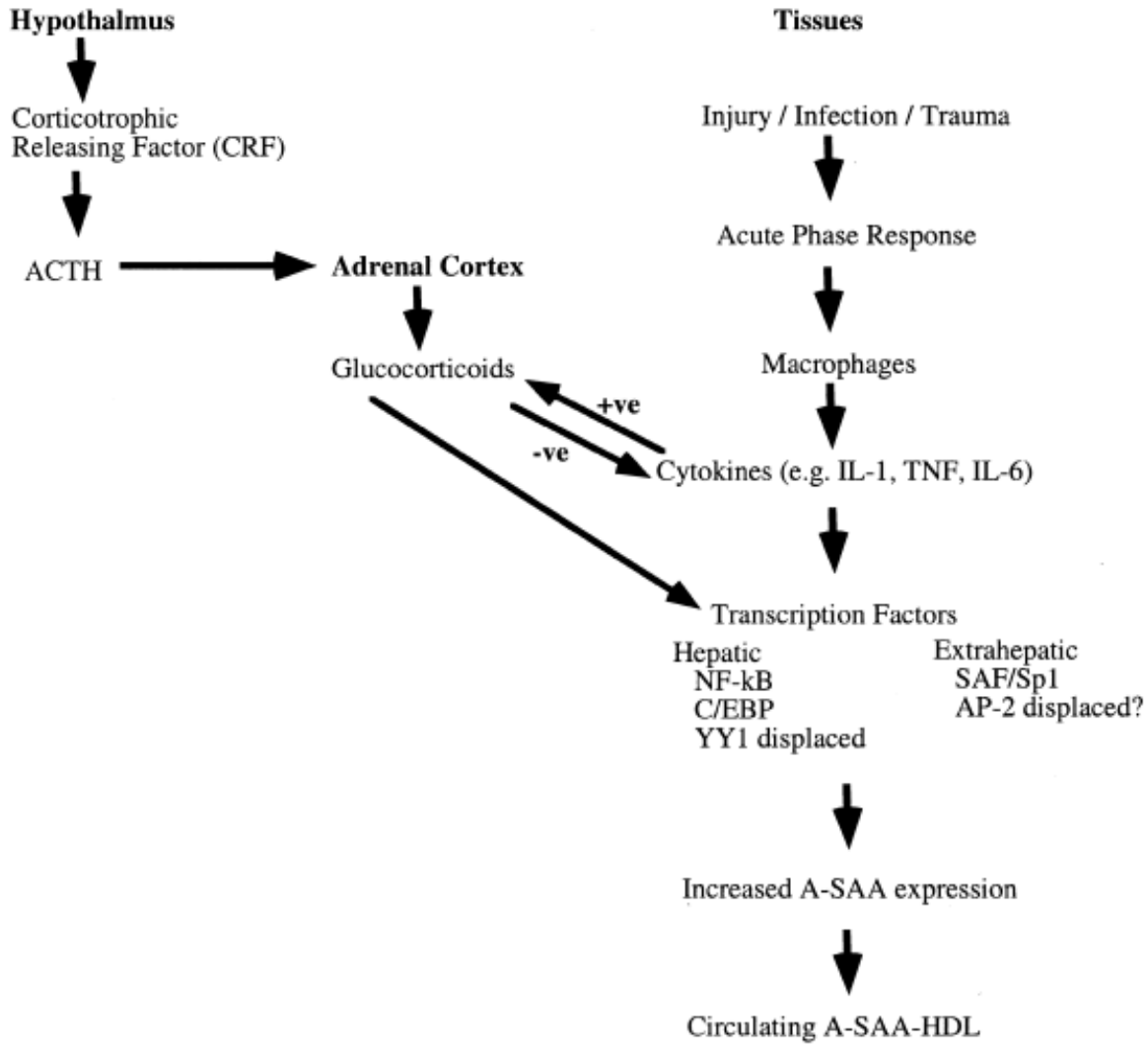

Figure 6: Flow diagram of A-SAA induction during acute-phase response. Inflammatory stimuli as triggers of acute-phase response lead to recruitment of macrophages and cytokine production which results in transcription factor changes and in succession to an increase expression of A-SAA. Glucocorticoids are able to enhance synthesis of A-SAA and are up regulated by various cytokines. [adapted from (72)]

1.4.3 Cystatin

Cystatin C, a cysteine-protease inhibitor is a protein of low molecular mass consisting of 120 amino acids (75). Renal glomerula filter the endogenous 13 kDa protein, cells of the proximal tubule take care of reabsorption and catabolisation but only small amounts are excreted into the urine (76). Therefore and also because it seems less affected by muscular mass, cystatin C is considered a

potentially reliable asset to serum creatinine and could be used (alone and with creatinine) for estimation of GFR and assessment of renal function (77).
A recent study dealing with acute kidney injury in critically ill patients with sepsis showed that plasma as well as urinary cystatin C levels might be useful predictors for septic AKI (78).

1.4.4 Neutrophil Gelatinase-Associated Lipocalin

Siderophores are small molecules synthesised and secreted by microorganisms like bacteria and fungi to scavenge iron from the environment only to be taken up again from the microorganisms to provide iron for essential life processes. Neutrophil gelatinase-associated lipocalin (NGAL), known to be expressed and produced by immune cells, tubular cells of the kidney and hepatocytes in various pathologic conditions as a mediator in iron traffic is attributed with bacteriostatic effects due to capturing and depletion of siderophores. NGAL is a protein with 21 kDa of weight and part of the lipocalin superfamily, therefore also carries the name lipocalin-2. Moreover, NGAL helps multiple cell types in growing and differentiation, including renal epithelia. Some of its functions even seem to be encouraged in the presence of iron complexes of siderophores. Accordingly, the synthesis of NGAL is suspected to be enhanced not only during the acute-phase response but also after injuries to the kidney. (79,80)

One study investigating NGAL plasma levels in critically ill patients found an association between elevation and sepsis independent of the state of renal dysfunction. The cut-off value at 98 ng/mL to discriminate sepsis from systemic inflammation without infection showed a sensitivity of 77% and specificity of 79%.(81)
Another report showed higher NGAL levels in non-AKI patients with sepsis compared to non-AKI patients without sepsis. The presence of sepsis, however, did not affect the diagnostic accuracy of NGAL for acute kidney injury - it only increased the optimal cut-off values.(82)
Various reviews also suggest a potentially important future role for NGAL in the diagnosis and prognosis of acute kidney injury (83,84).

1.4.5 Interleukin-10

First studies on interleukin-10 (IL-10) described its ability to inhibit the activation and appropriate function of various immune cells like T-cells, monocytes and macrophages. Characterised as an anti-inflammatory cytokine it is also known as human cytokine synthesis inhibitory factor (CSIF). The IL-10 protein is encoded by the eponymous gene consisting of 5 exons, located on chromosome 1, which arranges its synthesis out of 178 amino acids. The main purpose of IL-10 seems to be the limitation and ultimate termination of inflammatory response added by the task to help the differentiation and regulation of numerous immune cells like B-cells, NK-cells, cytotoxic- and helper-T-cells, granulocytes, mast cells, keratinocytes, dendritic cells and endothelial cell. The impact of IL-10 on the function and differentiation of regulatory T-cells appears to be crucial in the quest to control and regulate immune response and tolerance. Additionally, IL-10 inhibits the synthesis of other regulatory or pro-inflammatory cytokines such as interferon gamma or IL-2.(85-87)

While IL-10 is not commonly used as a laboratory marker to identify patients with sepsis, several studies found IL-10 capable of being a reliable biomarker for the prediction of mortality in patients with sepsis (88-90). One of them showed significantly higher IL-10 levels in patients who deceased within 48 hours (89), another one with a study collective of 59 patients stated that elevated IL-10 serum concentrations (>7,8 pg/mL) were able to identify all 8 deceased patients whereas no patients with normal IL-10 levels among the study population died (90).

1.4.6 Fibronectin

Fibronectin is a glycoprotein of the extracellular matrix which binds to integrins. These are transmembrane receptors that are involved in the attachment between cells and its surroundings. Like integrins, fibronectin is able to bind components of the extracellular matrix such as fibrin, collagen, proteoglycans or heparan sulphate. Two almost identical protein monomers linked by disulfide bonds form the protein dimer fibronectin. Despite being encoded by a single gene, alternative splicing of the pre-mRNA leads to the synthesis of several isoforms. In vertebrates

two types of fibronectin exist: soluble plasma fibronectin [~300 µg/mL (91)] produced by hepatocytes and insoluble cellular fibronectin as component of the extracellular matrix which is mainly produced by fibroblasts. Apart from cell adhesion, fibronectin plays a major role in cell growth, migration, differentiation and (plasma fibronectin) notably in wound healing. At the site of injury it is deposited along with fibrin forming blood clots. Decreased levels of plasma fibronectin are associated with acute inflammation but also recent surgical trauma and disseminated intravascular coagulation.(91-94)

A recent case-control study found significantly lower plasma levels of fibronectin (288,97 ± 89,10 mg/L) in patients with sepsis compared to patients with infectious diseases other than sepsis (341,24 ± 110,53 mg/l) and considered fibronectin a potentially reliable marker for sepsis (95).
Further data on plasma fibronectin confirmed significantly diminished levels in patients with sepsis by comparing plasma fibronectin of patients with sepsis (median 102 mg/L) to patients with fever who did not fulfil sepsis criteria (median 185 mg/L), patients with non-infectious diseases (median 175 mg/L) and a healthy control group (median 256 mg/L) (96).
Another study concerning catheter sepsis also found significantly decreased levels in septic patients compared to patient groups without sepsis (97).
Investigations among infants also report a significant depression of plasma fibronectin during sepsis (98,99).

1.4.7 Thrombomodulin

Encoded by the THBD gene with a molecular mass of 74 kDa and also recognised as CD141, thrombomodulin is an integral membrane protein on the surface of endothelial cells, mesothelial cells, monocytes and dendritic cells. By forming a 1:1 stoichiometric complex with thrombin, it works as a cofactor in the thrombin-induced activation of protein C. As the thrombomodulin-thrombin complex accelerates the activation of protein C thousandfould but at the same time acts in favour of coagulation by cleaving the thrombin-activatable fibrinolysis inhibitor into the active form, thrombomodulin appears to have an important role in the homeostasis of coagulation and fibrinolysis. In addition to binding thrombin,

thrombomodulin on the surface of endothelial cells seems to inactivate C3b, hence negatively regulates the complement system.(100-106)

One research study among neonates with sepsis stratifying study groups according to neonatal critical illness score revealed significantly higher levels of plasma thrombomodulin in case of sepsis compared to the control group and assessed thrombomodulin as possible biomarker for the evaluation of severity and prognosis of neonatal sepsis (107).
Research on adult patients found that higher thrombomodulin levels were associated with disseminated intravascular coagulation and multiorgan dysfunction syndrome (MODS) in septic patients. Thebiomarker might therefore be capable to assess the severity of sepsis as well as predicting MODS development (108,109).

1.4.8 Biotin

Biotin (also known as vitamin H) is a water-soluble coenzyme and part of the B-vitamin family (vitamin B_7). It has long been recognised as an important enzyme required in central metabolic processes such as biotin-dependent carboxylases and decarboxylases and is involved in the synthesis of fatty acids, catabolism of amino acids with branched chains and may help to maintain steady blood sugar levels. Apart from metabolism it is also involved in biotin-dependent transcription regulation and is not only used in human organisms but it is an essential cofactor in various microorganism, in particular in bacteria.(110-114)
Although biotin-deficiency has been linked to several pathologic conditions such as loss of hair, severe dermatitis and loss of muscular coordination, no data on biotin as a diagnostic marker for inflammation like SIRS or sepsis is available.(114)

1.4.9 Substance P

The neuropeptide substance P which was discovered in 1931 acts as neurotransmitter and neuromodulator. It is a peptide that comprises 11 amino acids and belongs to the neuropeptide family of tachykinines. Substance P can be found in the brain and spinal cord and is released from the terminals of specific

sensory nerves in response to inflammatory processes and pain. Besides it is not only involved in the stimulation of cell growth but is also regarded as a potent vasodilator.(115-119)

Additionally substance P is supposed to be released from nerve endings in various tissues during inflammatory states like asthma, immune-complex-mediated lung injury, experimental arthritis, and inflammatory bowel disease and seems to increase the permeability of small blood vessels which promotes plasma paravasation. Therefore, the peptide was classified as pro-inflammatory agent.(120)

Serum level tests of substance P among patients with postoperative sepsis revealed elevated levels compared to controls, but significant differences between survivors and patients who died in the course of the condition were only witnessed during the final phase of sepsis.(121)

Complementary data on plasma levels, however, claim that substance P is not increasingly released during course of human sepsis and septic shock.(122)

2 Methods

2.1 Study Objectives

2.1.1 Primary Objectives

The primary study objective was to evaluate automated multiplexed combined biomarkers measured with Hyborg Dx RED (Anagnostics Bioanalysis GmbH) as early predictors of positive blood cultures in patients with SIRS. Investigated biomarkers were IL-8, SAA, cystatin C, NGAL, IL-10, fibronectin, thrombomodulin, biotin and substance P. Therefore, the 0-Hypothesis is: levels of these biomarkers lack the ability to predict positive blood cultures in patients presenting with SIRS, hence cannot differentiate between SIRS and sepsis. The alternative hypothesis is, that biomarkers investigated with this multiplexed analysis system are able to predict positive blood cultures in patients with SIRS and allow the differentiation between SIRS and sepsis.

2.1.2 Secondary Objectives

In further investigations also routinely with Cobas 8000 system (Roche Diagnostics) measured biomarkers (CRP, PCT, IL-6, suPAR and creatinine) were evaluated alongside biomarkers of the Hyborg device:

- Examination of the diagnostic accuracy of CRP, PCT, IL-6, suPAR and creatinine to differentiate between SIRS and sepsis and therefore the ability to predict positive blood cultures
- Evaluation of the prognostic potential of all biomarkers in terms of the 30-day mortality
- Comparison of all biomarkers in different study groups and analysis of possible associations between plasma levels and causative pathogens.
- Correlation between biomarker levels and duration of patients' hospitalisation.
- Assessment of diagnostic and prognostic potential of combinations of biomarkers

2.2 Study Design and Data & Sample Collection

This cohort study was conducted between January 2012 and July 2013 at the Department of Medical and Chemical Laboratory Diagnostics and the Department of Pulmonary Medicine, Medical University Graz.

Patients presenting with symptoms of systemic inflammatory response syndrome were screened for study inclusion. Blood samples were taken at first contact with the patients at the emergency department, or at the wards the patients were transferred to and some hundred micro litres of plasma were aliquoted, frozen and stored at – 80 °C right after blood sample drawing for further testing. Additionally three pairs of blood cultures were taken from each patient, incubated for a maximum of seven days (BACTECs blood culture system; Becton Dickinson, Cockeysville, MD) and evaluated at the Microbiological Laboratory, Department of Internal Medicine and the Bacteriological Laboratory, Institute of Hygiene, Medical University Graz.

Inclusion criteria were as follows:

- Patients above 18 years of age
- Clinical suspicion for bacteremia/septicemia
- SIRS criteria met
- In case of negative blood cultures: no antibacterial treatment during past 5 days before blood samples were taken
- Given informed consent

Causative Pathogens of infections detected in the blood stream were as follows:

- *Staphylococcus aureus*
- *Escherichia coli*
- *Candida* species

Septicemia was defined as the presence of at least one positive blood culture. Bacteremia and fungemia meant the existence of bacterial, respectively fungal infection and their proof in blood cultures. Therefore the terms septicemia, or more specifically bacteremia and fungemia implied sepsis whereas their absence meant

the condition of systemic inflammatory response syndrome did not come from an infection.

For statistical analysis, the study population was divided in five groups:

(1) SIRS and *Staphylococcus aureus* infection
(2) SIRS and *Escherichia coli* infection
(3) SIRS and fungemia (*Candida* spp.)
(4) SIRS and septicemia (*Staph. aureus* or *E.coli* or *Candida* spp.)
(5) SIRS but negative blood cultures

On receipt, not later than one hour after collection, serum and whole blood samples, routine infection laboratory biomarker, being CRP, PCT and IL-6 as well as creatinine were determined by Cobas 8000 system (Roche Diagnostics, Rotkreuz, Switzerland). Moreover glomerular filtration rate (GFR) was calculated using the MDRD formula (Modification of Diet in Renal Disease) (123). Impaired renal function was defined under a GFR cut-off value of 50 mL/min.

Serum concentrations of suPAR were measured retrospectively using suPARnostic^{TM} ELISA kit (ViroGates, Copenhagen, Denmark).

Clinical data for further characterisation of patient database (age, gender, outcome, 30 days mortality, days hospitalised after study inclusion) was collected using MEDOCS software system. The outcome was divided in 3 groups comprising patients who recovered, died within 24 hours after presentation or later events of death which was therefore considered as natural study endpoints.

2.3 Hyborg Dx RED and Hybcell Technology

The identification of the exact concentrations of biomarkers (mentioned in the sections above) in a blood sample with the multiplex analysis system we used, more specifically the Hyborg Dx RED device (Anagnostics Bioanalysis GmbH, Sankt Valentin, Austria), is based on the principle of a competitive immunoassay or a sandwich immunoassay.(124)

Competitive assay: Specific conjugates are mixed with the analysis sample (here: plasma) which is then filled into small cylinders, called hybcells. The surface of the

hybcells is coated with immobilised antibodies. The biomarkers in the sample – which amounts are to be determined – compete with antigens in the conjugates for binding to the antibodies. The more analyte (biomarker) present in the sample, the less competitor antigens from the conjugates bind to the antibodies. Next, the unbound competitor antigens are washed off the hybcell in a cleaning step. Since the competitor antigens are equipped with fluorescence, the measured signal decreases by the amount of analyte in the sample.(124)

Sandwich immunoassay: Biomarkers in the sample bind to monoclonal antibodies on the hybcell surface. Specific fluorescent antibodies from the conjugate bind to the analytes. The more analyte in the sample, the more are captured with fluorescent antibodies and the greater is the fluorescence signal on the surface.(124)

Of every patient's blood sample 100 µL of defrosted plasma (stored at – 80 °C) were aliquoted. Each sample needs one hybcell and one added conjugate to be measured. Eight hybcells (8 samples) can be allocated on a rack at once, which is then inserted into the device. With all eight possible sample spaces on the rack taken, the device needs approximately 21 minutes for measurement of all biomarkers in all eight blood samples.(124)

Analyte	Reference ranges	Analytical measuring range	Units
Biotin	~ 200	10 / 2.000	ng/L
Cystatin C	~ 0,6 – 1,5	0,5 /10	µg/mL
Fibronectin	200 – 450	10 / 500	µg/mL
IL-8	< 20	200 / 10.000	pg/mL
IL-10	< 0.5	1 / 10	ng/mL
NGAL (lipocalin-2)	~ 30	5 / 500	ng/mL
Serum amyloid A (SAA)	< 5	1 / 5000	µg/mL
Substance P (tachykinine)	< 1	0,5 / 50	ng/mL
Thrombomodulin	~ 1,5	1,5 / 500	ng/mL

Table 3: Biomarkers and performance characteristics of the Hyborg Dx RED [adapted from (124)].

2.4 Data Analysis

Statistical analysis of all data was carried out using SPSS, version 21 (SPSS Inc., Chicago, Illinois, USA). Medians and inter quartile ranges (IQR, 25%-75% quartile), respectively means and standard deviation are illustrated for continuous data whereas categorical data is depicted as proportions. For statistical purposes, biomarker levels below detection limit of the laboratory equipment (Cobas 8000 and Hyborg Dx RED) were set to zero; levels above were postulated as the actual maximum detection limit value. Kolmogorov-Smirnov and Shapiro-Wilks test were performed to assess normal distribution of the plasma concentration of biomarkers. Looking for significant differences, all five patient groups were compared with Kruskal Wallis test. Additionally, each two patient groups were compared separately using Mann-Whitney U test but p-values were not corrected for multiple comparisons and are therefore only descriptive. For statistical significance a p-value of less than 0,05 was required. Correlation between biomarkers and days hospitalized was explored with Spearman's rank correlation coefficient (Spearman's rho, r_s).

Biomarkers and combinations were further investigated using receiver operating characteristics (ROC, ROC curve analysis) and area under the curve (AUC) values are shown including a 95% confidence interval (CI). In the attempt to predict septicemia with biomarkers, Youdens Index was utilised to determine cut-off values and by implication sensitivity and specificity were calculated at a maximised Youdens Index.

Binary logistic regression analysis was performed with explanatory variables being considered significant at $p<0.20$ in the multivariable logistic model. In a next and final step, variables fulfilling this significance level were included in the forward stepwise procedure. The criteria for a variable to enter this final model was $p\leq0,05$. Variables were removed, if p-values exceeded 0,10. Results are displayed as odds ratios (OR) including a 95% confidence interval.

This study was accomplished corresponding to the Declaration of Helsinki, 1996, Good Clinical Practice and authorised by the local ethics committee, Medical University Graz, Austria (21-469 ex 09/10).

3 Results

3.1 Patient Database and Baseline Characteristics

One hundred fifty-nine patients met inclusion criteria (all of whom febrile T>38°C). The gender distribution of the entire population was reasonably balanced (78 female and 81 male patients) which equally applies to their distribution in study groups 0 to 4. One hundred forty-eight patients needed hospitalisation whereas all patients underwent a 90-days follow-up for a complete outcome evaluation. Cumulative demographic data is shown in Table 4, demographic data classified according to blood culture/causative pathogen in Table 5 and distribution of study population into groups in Figure 7.

Patients included	n	159
Blood culture	negative	49
	positive	110
Gender	female	78
	male	81
Age [years]	mv	66
	SD	15
Days hospitalized after study inclusion	n	148
	mv	15
	SD	15
Outcome	n=alive	123
	n=deceased within 48 hours	8
	n=eventual deaths	36
30-days mortality	n	27

Table 4: Demographic data of study population depicted as numbers counted (n), mean values (mv) and standard deviation (SD).

	group 0	group 1	group 2	group 3	group 4
	SIRS without septicemia	*Staph. aureus*	*E. coli*	*Candida spp.*	Overall septicemia (group 1+2+3)
Patients	49	49	51	10	110
Age [years]	65 ± 18.1	64,5 ± 13,4	67,7 ± 13,9	63,3 ± 5,7	65,9 ±13,2
Gender [f/m]	19 / 30	22 / 27	31 / 20	6 / 4	59 / 51
Days hospitalized	11,9 ± 12	21,8 ± 17,4	12,5 ± 12,9	18,5 ± 13,3	17,3 ± 16,6
Alive	45	31	40	7	78
Died within 48 hours	2	2	3	1	6
Eventual death	4	18	11	3	32
30-days mortality	3	11	10	3	24

Table 5: Numbers respectively means ± standard deviation of demographic data related to classification into study groups based on blood culture results.

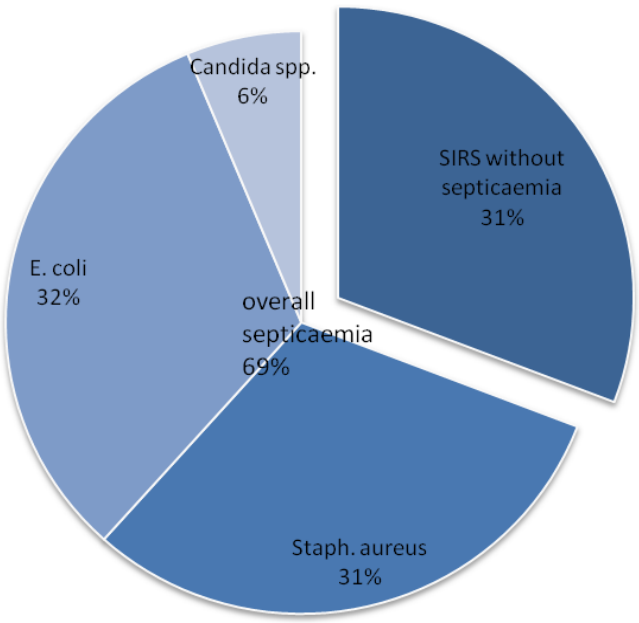

Figure 7: Classification according blood culture results. Among positive blood cultures (septicemia) *Staphylococcus aureus*, *Escherichia coli* and *Candida species* were found.

Impaired renal function as function of glomerular filtration rate was found in more than half of the patients with positive blood cultures, except for *Candida* infected patients (n=10; 40%) whereas patients without blood stream infection according to blood cultures showed a GFR below 50 mL/min to an even lesser extent (n=49; 36,73%). Numbers and rates of impaired renal function are depicted in Table 6 and Figure 8.

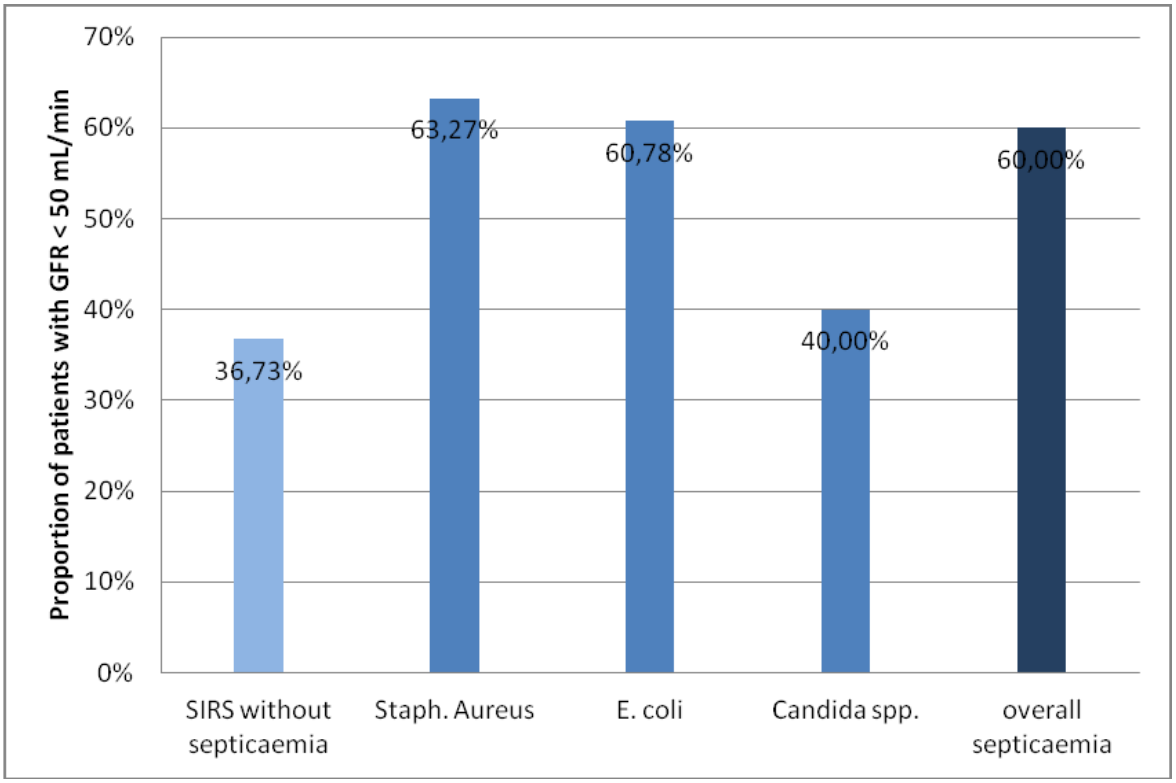

Figure 8: Proportion of patients with impaired renal function (GFR < 50 mL/min) sorted in study groups.

Figure 9 shows the outcome rates found in the five study groups. The outcome was differentiated between patients who were alive after the follow-up period, patients who died within the first 48 hours after study inclusion and patients who eventually died within the follow-up period of 90 days.

The highest mortality rate came upon study group 1 (36,73%), whereas patients without septicemia (group 0) had the greatest survival rate (91,84%). Comparing mortality rates of patients with positive and negative blood cultures (groups 4 and

0), events of death in patients with septicemia (29,09%) occurred a lot more often than in patients with no infection but SIRS (8,16%).

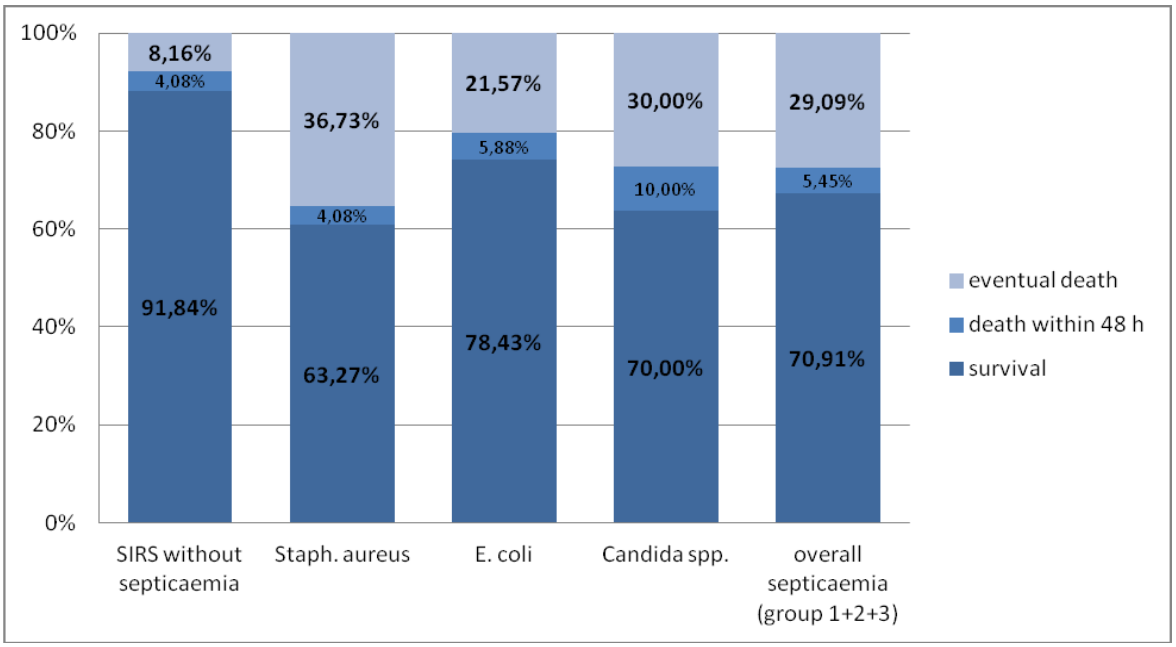

Figure 9: Outcome depicted as percentage of total group population.

The data of all biomarkers and glomerular filtration rate, arranged in order of groups according to causative pathogen is displayed in Table 6 (plasma levels of biomarkers measured with electrochemiluminescence immunoassays on the Cobas 8000 system) and Table 7 (biomarkers quantified using Hyberg Dx RED multiplex analysis system).

			group 0	group 1	group 2	group 3	group 4	
			SIRS without septicemia (n=49)	*Staph. aureus* (n=49)	*E. coli* (n=51)	*Candida* spp. *(n=10)*	Overall septicemia (n=110)	p-values comparing group 0 and 4
CRP [mg/L]	n=158	median	67	134	144	85	134	0,086
		IQR	29 – 231	52 – 256	54 – 252	32 – 183	52 – 252	
PCT [ng/mL]	n=155	median	0,34	1,40	1,84	0,93	1,53	<0,001
		IQR	0,12 – 1,18	0,29 – 7,93	0,42 – 11,30	0,11 – 1,95	0,32 – 9,12	
IL-6 [pg/mL]	n=153	median	153	202	360	143	216	0,054
		IQR	81 – 279	110 - 448	113 - 1320	60 - 365	110 – 851	
suPAR [ng/mL]	n=159	median	5,83	8,84	7,23	9,94	8,36	0,001
		IQR	4,63 – 7,04	5,67 – 13,99	4,30 – 11,30	9,43 – 12,01	5,06 – 12,49	
Creatinine [mg/dL]	n=148	median	1,15	1,63	1.29	0.95	1.50	0,169
		IQR	0,97 – 1,77	1,08 – 3,75	1,01 – 2,07	0,62 – 1,93	0,99 – 2,61	
GFR<50 [mL/min]	n=148	n	18	31	31	4	66	

Table 6: Medians and interquartile ranges (IQRs) of routinely tested biomarkers in patients with SIRS (measured with Cobas 8000 system, Roche Diagnostics, Rotkreuz, Switzerland); patients with a GFR < 50 mL/min (numbers shown) were considered to have impaired renal function. P-values below 0,05 (using Mann-Whitney U test) show statistically significant higher infection marker levels in patients with positive blood cultures (group 4) compared to those with negative blood cultures (group 0).

			group 0	group 1	group 2	group 3	group 4	
			SIRS without septicemia (n=49)	*Staph. aureus* (n=49)	*E. coli* (n=51)	*Candida* spp. (n=10)	Overall septicemia (n=110)	p-values comparing group 0 and 4
IL-8 [pg/mL]	n=158	median	0	0	597	357	451	0,009
		IQR	0 – 499	0 – 652	379 – 794	0 – 1050	0 – 756	
SAA [µg/mL]	n=159	median	700	700	700	232	700	0,666
		IQR	174 – 700	320 – 700	245 – 700	59 – 700	222 – 700	
Cystatin C [µg/mL]	n=159	median	1,69	2,26	1,93	1,64	2,06	0,117
		IQR	1,33 – 2,63	1,58 – 4,14	1,40 – 2,67	1,05 – 2,69	1,52 – 3,13	
NGAL [ng/mL]	n=159	median	17	37	23	29	28	0,046
		IQR	9 – 42	10 – 89	9 – 78	8 – 56	10 – 83	
IL-10 [ng/mL]	n=159	median	0,42	0,53	0,41	0,41	0,42	0,875
		IQR	0,09 – 2,19	0,12 – 1,73	0,16 – 1,77	0,05 – 1,11	0,12 – 1,73	
Fibronectin [µg/mL]	n=159	median	409	396	359	302	373	0,020
		IQR	352 – 444	355 – 436	306 – 420	204 – 343	311 – 433	
Thrombomodulin [ng/mL]	n=158	median	0,00	0,00	0,00	0,00	0,00	0,262
		IQR	0 – 0	0 – 0,85	0 – 1,15	0 – 0	0 – 0,85	
Biotin [ng/L]	n=155	median	21	43	93	187	71	0,004
		IQR	3 – 51	10 – 333	11 – 261	20 – 602	11 – 281	
Substance P [ng/mL]	n=159	median	0,30	0,31	0,38	0,33	0,33	0,633
		IQR	0,24 – 0,44	0,23 – 0,44	0,24 – 0,51	0,3 – 0,38	0,23 – 0,47	

Table 7: Medians and IQRs of biomarkers measured with Hyborg Dx RED device (Anagnostics Bioanalysis GmbH, Sankt Valentin, Austria); p-values according to Mann-Whitney U test depicted as in Table 6.

3.1.1 Biomarkers of Multiplex Analysis System

Comparing all study groups 0 to 4 (negative blood culture vs. *Staph.aureus* vs. *E.coli* vs. *Candida* spp.) with the Kruskal-Wallis test, p-values differed significantly in IL-8 (p=0,002; n=158), fibronectin (p<0,001; n=159) and biotin (p=0,028; n=155).

Looking at the infection markers measured with the multiplexed analysis system (Hyborg Dx RED device), patients with positive blood cultures showed significantly higher levels of IL-8 (p=0,009; median 451 pg/mL; IQR 0 – 756 pg/mL; 110 patients), NGAL (p=0,046; median 28 ng/mL; IQR 10 – 83; 110 patients) and biotin (p=0,004; median 71 ng/L; IQR 11 – 281; 108 patients) than patients whose blood cultures were negative [IL-8 (median 0 pg/mL; IQR 0 – 499; n=48), NGAL (median 17ng/mL; IQR 9 – 42; n=49), biotin (median 21 ng/L, IQR 3 – 51; n=47)].
In contrast, fibronection showed significantly lower levels in septic plasma samples (p=0,020; median 373 µg/mL; IQR 311 – 433; 110 patients) than in samples where blood cultures did not detect any pathogens (median 409 µg/mL; IQR 352 – 444; n=49). No significant differences in plasma levels comparing positive and negative blood culture specimens were found for SAA, cystatin C, IL-10, thrombomodulin and substance P.

Cystatin C increased significantly only in patients with Staphylococcus aureus infection (p=0,033; median 2,26 µg/mL; IQR 1,58 – 4,14; n=49) compared to group 0 (median 1,69 µg/mL; IQR 1,33 – 2,63; n=49). Among patients with sepsis, fibronectin showed significant differences between all groups except for group 0 compared to group 1. Fibronectin levels in patients with Staph. aureus (p=0,001 median 396 µg/mL; IQR 355 – 436; n=49) and *E. coli* infection (p=0,032; median 359 µg/mL; IQR 306 – 420; n=51) were significantly higher than in patients infected with *Candida* spp. (median 302 µg/mL; IQR 204 – 343; n=10) but still lower than in patients without blood stream infection. Additionally, fibronectin was significantly lower (p=0,010) in group 2 (*E. coli*) than in group 1 (*Staph. aureus*). In contrast to that, IL-8 showed higher levels in group 1 (p=0,006; median 0 pg/mL; IQR 0 – 652; n=49) compared to group 2 (median 597 pg/mL; IQR 379 – 794; n=51).

Comparisons of biomarker levels between study groups that are not mentioned above, particularly serum amyloid A, interleukin-10, thrombomodulin and substance P, showed no statistically significant differences.

As far as impaired renal function is concerned, levels of SAA (p=0,008), cystatin C (p<0,001), NGAL (p<0,001) and biotin (p<0,001) were elevated significantly compared to samples of patients with a glomerular filtration rate above 50 mL/min. Moreover, Spearman's rho revealed a highly significant correlation between plasma concentrations of creatinine and cystatin C (r_s=0,805; p<0,001) as well as NGAL (r_s=0,513; p<0,001), respectively between GFR < 50 ml/min and cystatin C (r_s=0,687; p<0,001) as well as NGAL (r_s=0,462; p<0,001).

On the other hand, IL-8, IL-10, fibronectin, thrombomodulin and substance P did not reveal significantly different plasma levels comparing patients with or without impaired renal function.

The relation between biomarkers and number of days hospitalised revealed only a slight correlation between increased levels of cystatin C (r_s=0,210; p=0,010) and longer duration of hospital stay.

3.1.2 Routinely tested Biomarkers

According to Kruskal-Wallis test PCT (p<0,001; n=155), suPAR (p=0,001; n=159) and creatinine (p=0,041; n=148), were significantly different comparing the four patient groups with no detected or different pathogens causing SIRS (negative blood culture vs. *Staph.aureus* vs. *E.coli* vs. *Candida* spp.).

Out of the routinely ordered biomarkers (measured with Cobas 8000 system), PCT (p<0,001; median 1,53 ng/mL; IQR 0,32 – 9,12; 106 patients) and suPAR (p=0,001; median 8,36 ng/mL; IQR 5,06 – 12,49; 110 patients) were significantly higher in patients with septicemia than in those with negative blood culture [PCT (median 0,34 ng/mL; IQR 0,12 – 1,18, n=49), suPAR (median 5,83 ng/mL; IQR 4,63 – 7,04, n=49)]. Differences in creatinine levels (positive vs. negative blood cultures) were not significant.

When going into detail of positive blood cultures, PCT was significantly higher in group 1 (p=0,003; median 1,40 ng/mL; IQR 0,29 – 7,93; n=49) and group 2 (p<0,001; median 1,84 ng/mL; IQR 0,42 – 11,30; n=49) than in group 0. SuPAR on the other hand was higher in group 1 (p=0,001; median 8,84 ng/mL; IQR 5,67 – 13,99) and group 3 (p=0,001; median 9,94 ng/mL; IQR 9,43 – 12,01) compared to patients with SIRS alone. Creatinine levels of patients with *Staph. aureus* infection (median 1,63 mg/dL; IQR 1,08 – 3,75; n=45) were significantly higher than levels in patients with *Candida* infection (p=0,034; median 0,95 mg/dL; IQR 0,62 – 1,93; n=10) as well as in patients without infection (p=0,018; median 1,15 mg/dL; IQR 0,97 – 1,77; n=49).

Plasma levels of CRP and IL-6 showed no significant differences at all between study groups. All other comparisons between biomarker plasma levels of different study groups that are not mentioned above showed no statistically significant differences.

Patients with impaired renal function showed higher levels of CRP, PCT, IL-6 and suPAR compared to patients with a GFR > 50 mL/min (all p-values were highly significant at p<0,001).

Spearman's rank correlation test found the association between days hospitalised and CRP (r_s =0,204; p=0,013) as well as suPAR (r_s=0,178; p=0,031) statistically significant with higher levels indication a longer hospital stay.

3.2 Diagnostic Accuracy of Biomarkers

3.2.1 Biomarkers of Multiplex Analysis System

Receiver operating characteristics (ROC) curve analysis in differentiating patients with sepsis from patients with SIRS without infection revealed the highest area under the curve (AUC) value of 0,646 for biotin (95% CI 0,550 – 0,743; n=155), followed by IL-8 with an AUC value of 0,625 (95% CI 0,534 – 716; n=158).

For the test results in the ROC curve analysis of fibronectin, the test direction was reversed as decreased plasma levels indicate rather a positive than a negative blood culture, which resulted in an AUC value of 0,616 (95% CI 0,524 – 0,708; n=159).

The ROC curves for biomarkers measured with the Hyborg device are displayed in Figure 10.

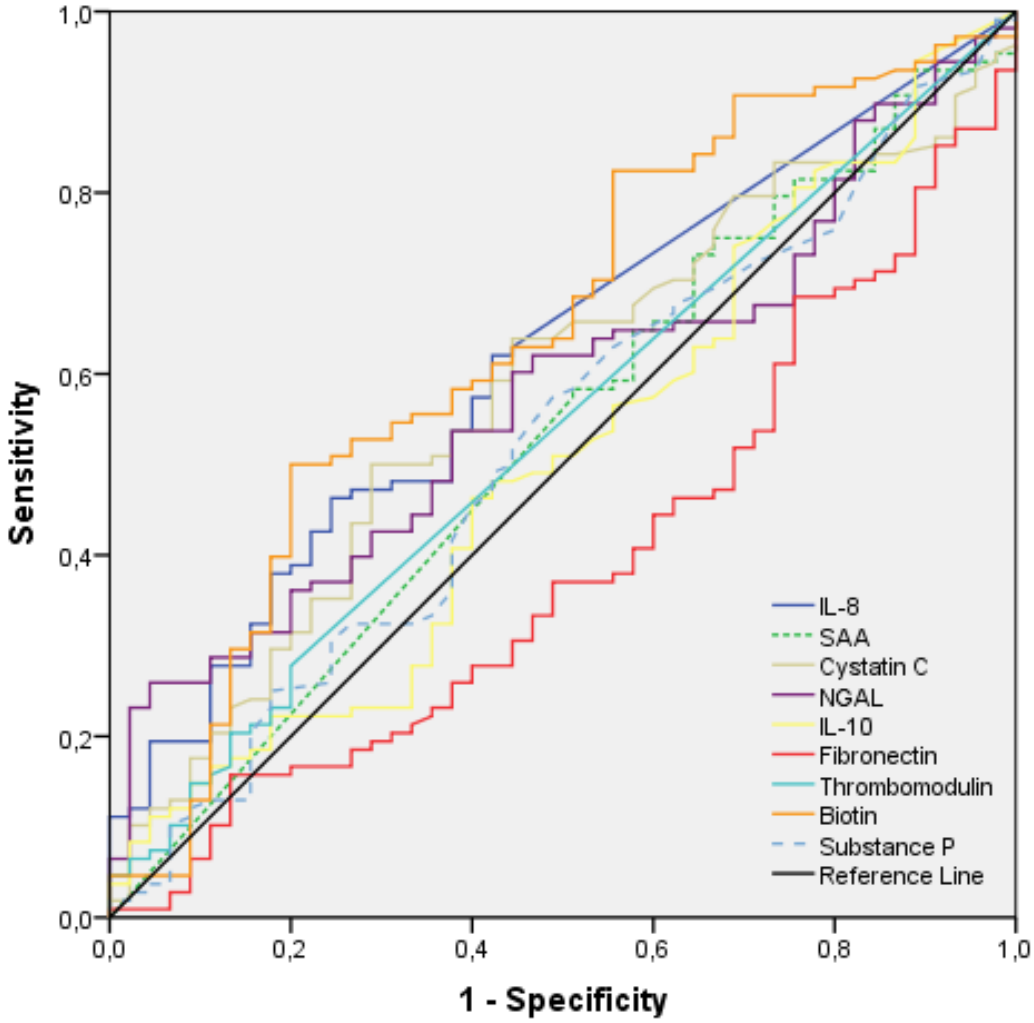

Figure 10: ROC curve analysis for biomarkers measured with Hyborg Dx RED device to distinguish between positive and negative blood cultures in patients with SIRS: interleukin-8, serum amyloid A, cystatin C, neutrophil gelatinase-associated lipocalin, interleukin-10, fibronectin, thrombomodulin, biotin and substance P.

For the prediction of sepsis in the study population, the optimal cut-off point for biotin was 70,4 ng/L with a sensitivity of 50% and a specificity of 81%. Sensitivity and specificity of IL-8 with a cut-off point at 507,2 pg/mL were 45% and 77%

respectively. Keeping in mind that fibronectin plasma levels showed a negative correlation with septicemia in SIRS patients, with a cut-off below 377,4 µg/mL indicating sepsis, sensitivity was 54% and specificity 69%.

All other AUC values were below 0,6 and are displayed together with cut-off values, sensitivity and specificity in Table 8.

Biomarker	AUC values	95% CI	Cut-off value (Youdens index)	Sensitivity	Specificity
IL-8 (n=158)	0,625	0,534 – 0,716	507,2 pg/mL	45	77
SAA (n=159)	0,519	0,422 – 0,617	289,4 µg/mL	73	35
Cystatin C (n=159)	0,578	0,484 – 0,672	2,1 µg/mL	50	71
NGAL (n=159)	0,599	0,509 – 0,689	82 ng/mL	26	96
IL-10 (n=159)	0,508	0,409 – 0,607	1,9 ng/mL	23	67
Fibronectin (n=159)	0,616	0,524 – 0,708	377,4 µg/mL	54	69
Thrombomodulin (n=158)	0,543	0,447 – 0,638	0 ng/mL	27	81
Biotin (n=155)	0,646	0,550 – 0,743	70,4 ng/L	50	81
Substance P (n=159)	0,524	0,426 – 0,622	0,3 ng/mL	56	53

Table 8: AUC values with 95% confidence intervals, cut-off values with associated sensitivity and specificity determined by maximised Youdens Index displayed.

3.2.2 Routinely tested Biomarkers

Out of the commonly used infection markers used in the quest to differentiate between patients with SIRS alone and patients with sepsis, PCT showed the highest AUC value of 0,694 (95% CI 0,607 – 0,781; n=155). SuPAR had an AUC

value of 0,660 (95% CI 0,575 – 0,745; n=159), whereas the rest exhibited lower AUCs: IL-6 0,597 (95% CI 0,593 – 0,692; n=153), CRP 0,586 (95% CI 0,488 – 0,683; n=158) and creatinine 0,570 (95% CI 0,476 – 0,663; n=148).
The ROC curves for routinely tested biomarkers are displayed in Figure 11.

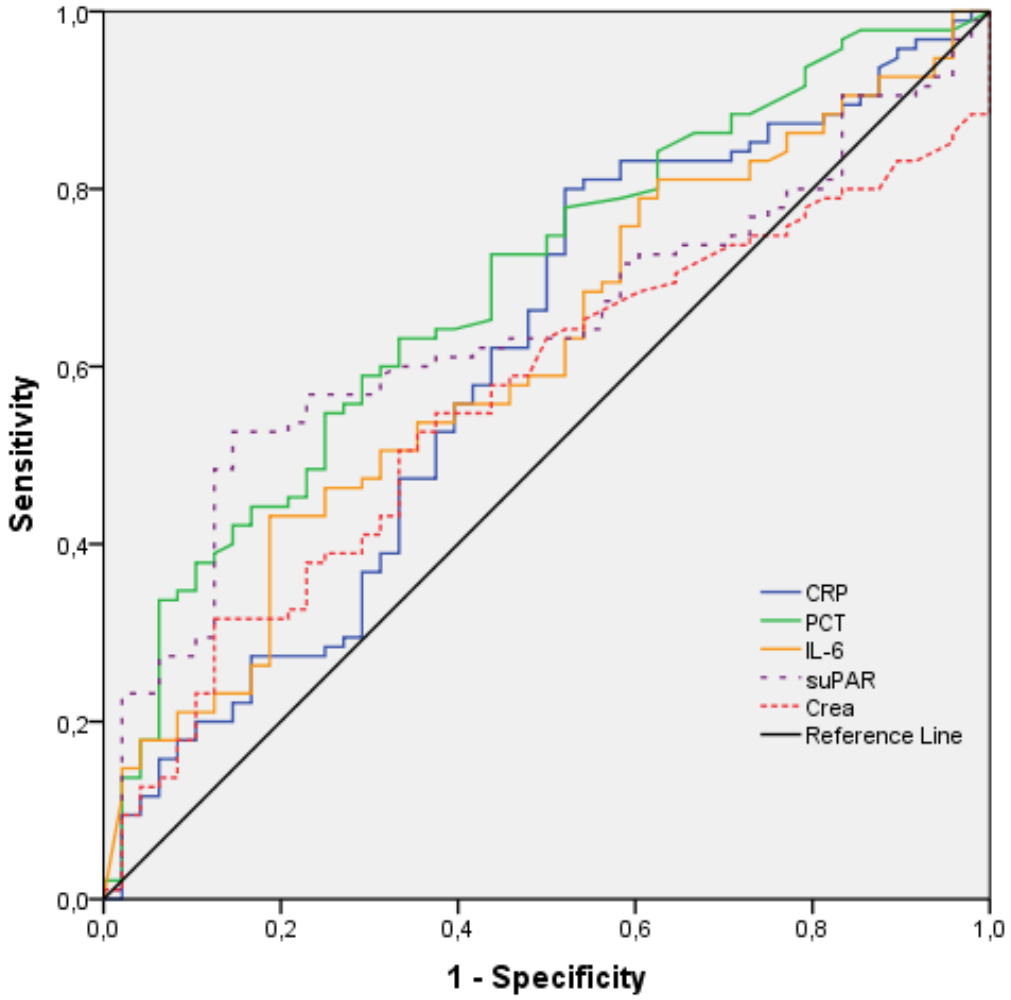

Figure 11: ROC curve analysis for routinely tested laboratory infection markers to distinguish between positive and negative blood cultures in patients with SIRS: C-reactive protein, procalcitonin, interleukin 6, soluble urokinase plasminogen activator receptor and creatinine.

A cut-off value of 0,6 ng/mL for PCT resulted in a sensitivity of 62% and specificity of 67% whereas suPAR's cut-off at 7,6 ng/mL had a sensitivity and specificity of 55% and 86% respectively. The optimal cut-off for IL-6 was at 303,1 pg/mL resulting in a sensitivity of 43% and specificity of 81%. The cut-off point for CRP was determined at 51,6 mg/L (sensitivity 75%; specificity 47) and for creatinine at

2,2 mg/dL (sensitivity 31%; specificity 88%). These results are summarised in Table 9.

Biomarker	AUC values	95% CI	Cut-off value	Sensitivity	Specificity
CRP (n=158)	0,586	0,488 – 0,683	51,6 mg/L	75	47
PCT (n=155)	0,694	0,607 – 0,781	0,6 ng/mL	62	67
IL-6 (n=153)	0,597	0,593 – 0,692	303,1 pg/mL	43	81
suPAR (n=159)	0,660	0,575 – 0,745	7,6 ng/mL	55	86
Creatinine (n=148)	0,570	0,476 – 0,663	2,2 mg/dL	31	88

Table 9: AUC values with 95% confidence intervals, cut-off values with associated sensitivity and specificity determined by maximised Youdens Index.

All 14 measured biomarkers were analysed in the binary logistic regression. SuPAR (p=0,188), IL-8 (p=0,014), NGAL (p=0,156), IL-10 (p=0,192) and fibronectin (p=0,096) were entered into the final model for stepwise binary logistic regression. Only suPAR and fibronectin prevailed in the final model as predictor of positive blood cultures, revealing an odds ratio (OR) for suPAR of 1,149 (95% CI and 1,046 – 1,262; p=0,004; n=158) and fibronectin OR of 0,994 (95% CI 0,988 – 0,999; p=0,014; n=158).

3.3 Prognostic Value of Biomarkers

3.3.1 Biomarkers of Multiplex Analysis System

The comparison of infection marker levels between the outcome groups of survival and death within thirty days presented three statistically significant results: IL-8 was significantly higher in the group of deceased patients (p=0,019; median 691 pg/mL; IQR 0 – 1114; n=27) than in the survival group (median 387 pg/mL; IQR 0

– 620; n=131). Patients who died within 30 days also showed higher cystatin C levels (p=0,043; median 2,7 µg/mL; IQR 1,6 – 3,4; n=27) compared to patients who survived (median 1,8 µg/mL; IQR 1,4 – 2,9; n=132). To a greater extent, higher biotin levels were associated with mortality within 30 days (p=0,009; median 133,2 ng/L; IQR 17,2 – 569,6; n=26) compared to survival of this period (median 27,0 ng/L; IQR 4,5 – 202,8; n=129).

All other biomarker levels did not show significant differences between patients who survived or died within the first 30 days after study inclusion and are depicted together with the other biomarkers in Table 10.

	Survived			Deceased (within 30 days)			p-value
	median	IQR	n	median	IQR	n	
IL-8 [pg/mL]	387	0 – 620	131	691	0 – 1114	27	0,019
SAA [µg/mL]	700	176 – 700	132	700	245 – 700	27	0,662
Cystatin C [µg/mL]	1,8	1,4 – 2,9	132	2,7	1,6 – 3,4	27	0,043
NGAL [ng/mL]	21,7	9,0 – 62,4	132	38,1	13,9 – 102,7	27	0,085
IL-10 [ng/mL]	0,48	0,11 – 2,08	132	0,28	0,10 – 1,64	27	0,604
Fibronectin [µg/mL]	392	329 – 437	132	367	320 – 433	27	0,444
Thrombomodulin [ng/mL]	0,00	0,00 – 0,00	131	0,00	0,00 – 2,28	27	0,084
Biotin [ng/L]	27,0	4,5 – 202,8	129	133,2	17,2 – 569,6	26	0,009
Substance P [ng/mL]	0,32	0,24 – 0,47	132	0,32	0,27 – 0,47	27	0,717

Table 10: Characteristics (median, inter quartile range and numbers of valid data) of novel multiplexed biomarkers to picture plasma level differences between patients who survived and patients who died within the first 30 days after study inclusion; p-values comparing the two groups of different outcomes were computed using Mann-Whitney U test.

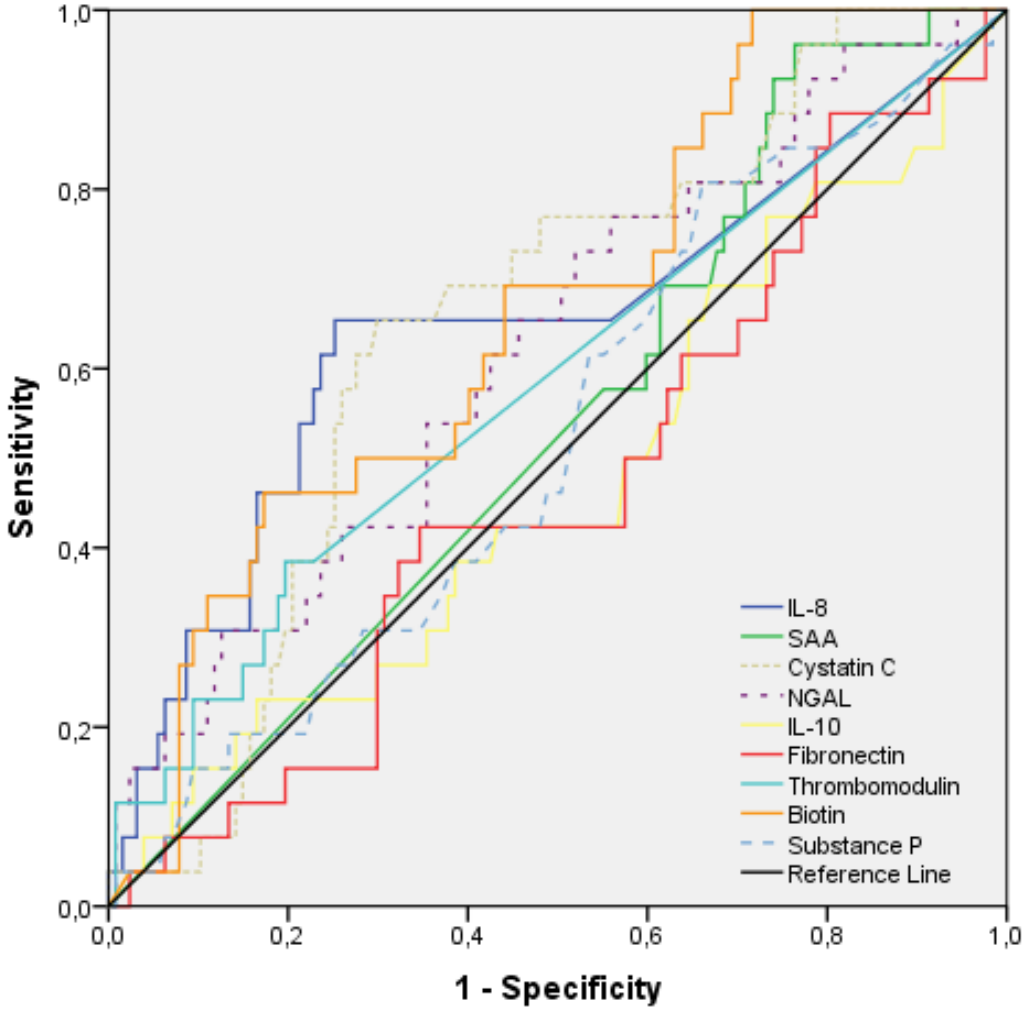

Figure 12: ROC curve analysis of novel multiplexed biomarkers to differentiate between survival and death within 30 days of study inclusion.

ROC curve analysis of 30-days mortality using novel multiplexed biomarkers (Figure 12) revealed the highest area under the curve (AUC) value of 0,663 for biotin (95% CI 0,555 – 0,771). IL-8 showed an AUC value of 0,637 (95% CI 0,507 – 0,767) followed by cystatin C with and AUC of 0,624 (95% CI 0,513 – 0,735).
All other AUC values and associated 95% confidence intervals for the prediction of 30 days mortality were lower and are displayed in Table 11.

	30-days mortality	
Biomarker	AUC value	95 % CI
biotin	0,663	0,555 – 0,771
IL-8	0,637	0,507 – 0,767
Cystatin C	0,624	0,513 – 0,735
NGAL	0,605	0,486 – 0,725
Thrombomodulin	0,580	0,455 – 0,705
Fibronectin	0,547	0,427 – 0,667
IL-10	0,532	0,409 – 0,655
SAA	0,524	0,413 – 0,636
Substance P	0,522	0,407 – 0,637

Table 11: AUC values and 95% CIs of Hyborg – inflammation markers for prediction of 30-days mortality revealed by ROC curve analysis

3.3.2 Routinely tested Biomarkers

Biomarker levels of suPAR were significantly higher in the group of patients deceased within 30 days (p=0,004; median 9,5 ng/mL; IQR 6,5 – 14,0; n=27) than in the survival group (median 6,6 ng/mL; IQR 4,6 – 10,3; n= 132). To a lesser extent PCT also showed increased levels in patients who died within 30 days (p=0,037; median 2,4 ng/mL; IQR 0,4 – 10,9; n=26) than in those who survived (median 0,7 ng/mL; IQR 0,2 – 3,8; n=129).

All other infection markers did not show significant differences regarding 30-days mortality are displayed together with the biomarkers outlined in Table 12.

	Survived			Deceased (within 30 days)			p-value
	median	IQR	n	median	IQR	n	
CRP [mg/L]	103,4	35,8 – 241,2	132	180,8	60,7 – 240,8	26	0,189
PCT [ng/mL]	0,7	0,2 – 3,8	129	2,4	0,4 – 10,9	26	0,037
IL-6 [pg/mL]	191	93 – 596	128	169	81 – 1135	25	0,925
suPAR [ng/mL]	6,6	4,6 – 10,3	132	9,5	6,5 – 14,0	27	0,004
Creatinine [mg/dL]	1,2	1,0 – 2,2	125	1,9	1,1 – 3,1	23	0,142

Table 12: Characteristics (median, inter quartile range and numbers of valid data) of routinely tested laboratory infection markers to picture plasma level differences between patients who survived and patients who died within the first 30 days after study inclusion; p-values comparing the two groups of different outcomes were computed using Mann-Whitney U test.

	30-days mortality	
Biomarker	AUC value	95 % CI
suPAR	0,676	0,572 – 0,781
PCT	0,630	0,516 – 0,744
Creatinine	0,597	0,466 – 0,727
CRP	0,582	0,474 – 0,689
IL-6	0,506	0,376 – 0,636

Table 13: AUC values and 95% CI of routinely tested biomarkers for the prediction of 30-days mortality, revealed by ROC curve analysis.

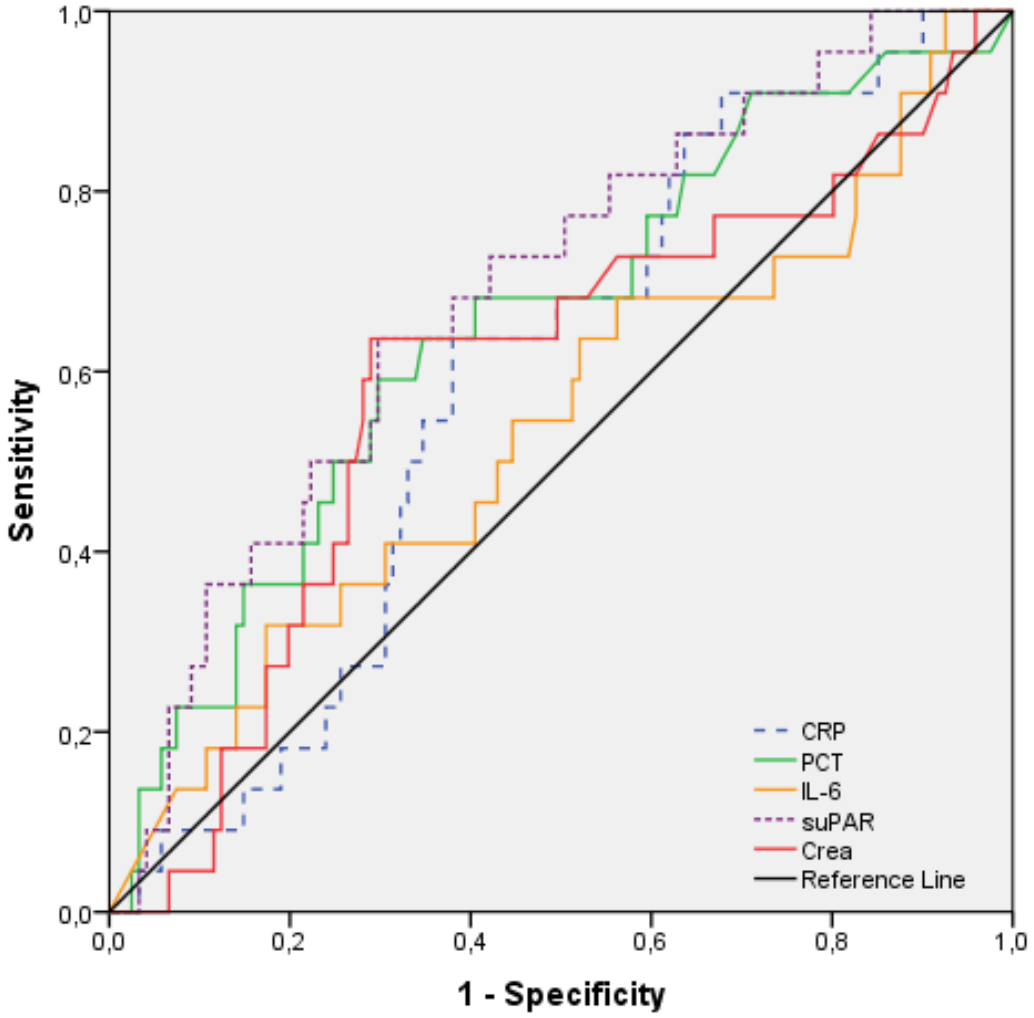

Figure 13: ROC curve analysis of routinely tested biomarkers to differentiate patients who died within 30 days of study inclusion and those who survived.

Among the routinely tested infection markers, ROC curve analysis of 30-days mortality (Figure 13) showed the highest AUC value of 0,676 (95% CI 0,572 – 0,781) for suPAR and PCT 0,630 (95% CI 0,516 – 0,744). AUC values for the other biomarkers were below 0,6 and are displayed in Table 13, together with biomarkers mentioned before.

Again, in the binary logistic regression all 14 biomarkers were evaluated as covariates for the prediction of 30-days mortality. IL-6 (p=0,110), suPAR (p=0,161), creatinine (p=0,019), IL-8 (p=0,010), cystatin C (p=0,017) and NGAL (p=0,199) were included in model. In the final stepwise binary logistic regression only IL-8 (OR 1,001; 95 % CI 1,000 – 1,001; p=0,067; n=143) and NGAL (OR 1,005; 95% CI 1,000 – 1,010; p=0,031; n=143) remained in the designed model.

4 Discussion

This present study focused on the investigation of selected laboratory markers (biomarkers) concerning their prognostic and diagnostic potential in a homogenous population with systemic inflammatory response syndrome.

As far as the distinction between sepsis and SIRS is concerned, we found that plasma levels of PCT, suPAR, IL-8, NGAL and biotin were significantly elevated in SIRS patients with positive blood cultures compared to those with negative blood cultures, whereas fibronectin levels were significantly diminished. Only two parameters showed significant differences between gram-positive and gram-negative bacteria: while IL-8 increased, fibronectin levels were lower in *E.coli*- compared to *Staph. aureus*- sepsis.

Additionally, fibronectin was also significantly diminished in patients with fungemia (*Candida* spp.) compared to patients with bacteremia. After all, the quest to distinguish between each blood culture results of patients with SIRS was best served by the observation of fibronectin plasma level reduction as differences between every group were significant, except between negative blood cultures and *Staph.aureus*: fibronectin levels were highest, when no blood stream infection was found, followed by gram-positive and gram-negative bacteremia and being lowest in fungemia. These findings are in line with the results of Mamani and colleagues (95), who found lower fibronectin plasma levels in patients with sepsis than in patients with infectious diseases other than sepsis. Although the work of Ruiz et al. (96) discovered substantially lower fibronectin levels in septic patients (median 102 mg/L) than this study (median 373 µg/mL), the biomarker showed a similar trend: plasma levels were lowest in patients with sepsis, followed by febrile patients without sepsis (median 185 mg/L), patients with non-infectious diseases (median 175 mg/L) and healthy patients (median 256 mg/L). However, implications have to meet with caution, considering the distinct overlapping of inter quartile ranges.

The well-established inflammation biomarker CRP admittedly tended to show greater elevations in the groups of positive blood cultures, but did not reveal any significant differences whatsoever among these groups, which on the other hand was not surprising as Carrigan et al. (6) also reviewed a significant overlap

between CRP concentrations in patients with sepsis and SIRS alone in different studies. The same insignificance applies to SAA, IL-6, IL-10, thrombomodulin and substance P.
Despite the fact, that creatinine is part of the definition of severe sepsis, as plasma levels twice as high as standard values indicate renal dysfunction (4), baseline creatinine levels showed no significant difference between positive and negative blood cultures.

As far as organ dysfunction is concerned, it has to be considered that impaired renal function, in this study defined as GFR < 50mL/min, was associated with elevated plasma levels of CRP, PCT, IL-6, suPAR, SAA, cystatin C, NGAL and biotin.
Especially the association between cystatin C and NGAL elevation and a lower GFR did not surprise, as Aydogdu and colleagues (78) found cystatin C to be a useful marker for the prediction of acute kidney injury in septic patients and reviews of Haase (84) and Clerico (83) found evidence on NGAL as biomarker for AKI. Highly significant correlations ($p<0,001$) between cystatin C, NGAL and creatinine, respectively GFR < 50 mL/min found in this study underline their findings.

Our investigation on the diagnostic accuracy for the prediction of septicemia found the best performance for PCT with an AUC of 0,694 (95% CI 0,607 – 0,781) according to ROC curve analysis, which acknowledges its supplementary use in the diagnosis of sepsis, recommended by the s-2 German Sepsis Guidelines (4). The study of Rowther and colleagues (3) among 99 critically ill patients (60 with sepsis) found a sensitivity of 100% for PCT to identify patients with sepsis but attributed the biomarker with the lack of specificity (61,66%). However, our figures to distinguish between sepsis and SIRS alone with the help of PCT (cut-off 0,6 ng/mL) showed lower sensitivity (62%) but a slightly higher specificity (67%).
The second best performance was accomplished by suPAR with an AUC of 0,660 (95% CI 0,575 – 0,745) and a reasonably good specificity of 86% at a cut-off of 7,6 ng/mL but low sensitivity (55%). Result of Hoenigl et al. (58) for suPAR showed a

higher AUC value (0,726; 95% CI 0,638 – 0,814) with a sensitivity of 62% and specificity of 77% but found a similar cut-off for suPAR at 7,9 ng/mL.

Biotin, for which up to now only very limited data have been published, showed the third highest AUC value of 0,646 (95% CI 0,550 – 0,743) in our study to predict septicemia. Biotin's sensitivity (cut-off value 70,4 ng/L) was low at 50% but its specificity was decent at 81%.

Previous studies on IL-8 investigating its diagnostic accuracy in predicting positive blood cultures were conducted on newborns and suggested it to be a promising asset for sepsis diagnosis. Santana and colleagues (70) tried to predict septicemia among 40 neonates with suspected infection with the help of IL-8 and found a high specificity (96%) but a limited sensitivity (62%). In our study specificity (77%) was not as high and sensitivity was even lower (45%) with an optimal cut-off value for IL-8 at 507,2 pg/mL. IL-8 showed the fourth best performance for predicting septicemia (AUC 0,625; 95% CI 0,534 – 0,716), respectively.

The best cut-off value for NGAL to distinguish between positive or negative blood cultures in SIRS patients was found at 82 ng/mL and accounted for a very promising specificity of 96%, which thrived at the expense of a very low sensitivity (26%). AUC value for NGAL was 0,599 (95% CI 0,509 – 0,689). Martensson et al. (81) already found NGAL helpful to identify bacterial infections in critically ill patients but used a higher cut-off value (98 ng/mL), which resulted in a higher sensitivity (77%) but lower specificity (79%).

IL-6 is already a commonly used, but not undisputed biomarker for the diagnosis of sepsis. Harbarth et al. (41) also investigated IL-6 as biomarker to discriminated between SIRS and sepsis and found an AUC value of 0,750 and the cut-off value at 200 ng/L accounted for a sensitivity and specificity of 67% and 72%. Jones and colleagues (51) also tried to use IL-6 to distinguish between SIRS and sepsis but found less promising results with a lower AUC value of 0,515 (cut-off 68,5 pg/mL; sensitivity 51%, specificity 52%). Our data could not resolve the controversial use of IL-6 in the diagnosis of sepsis, revealing an AUC value of 0,597 (95% CI 0,593 – 0,692) with a sensitivity and specificity of 43% and 81% (cut-off 303,1 pg/mL).

As mentioned above, the well-established inflammation marker CRP did not prevail in our study as indicator of sepsis in patients with SIRS. Our statistical

analysis on CRP revealed an AUC value of 0,586 (95% CI 0,488 – 0,683) with a sensitivity and specificity of 75% and 47% (cut-off 51,6 mg/L).

Furthermore, we investigated the prognostic value of all 14 biomarkers by analysing the plasma level differences between the group of patients that survived and the group of patients that deceased within 30 days after study inclusion. We found the most promising results for suPAR with an AUC value of 0,676 (95% CI 0,572 – 0,781), followed by biotin 0,663 (95% CI 0,555 – 0,771), IL-8 0,637 (95% CI 0,507 – 0,767) and PCT 0,630 (95% CI 0,516 – 0,744).
One study on 59 patients with *Staph. aureus* bacteremia conducted by Rose and colleagues (90) found the IL-10 threshold of 7,8 pg/mL able to identify all 8 patients who deceased. This very promising result on IL-10 as a prognostic biomarker could not be confirmed in our study as we found low AUC values of 0,532 (95% CI 0,409 – 0,655) for IL-10 to predict 30-days mortality.

When looking at the results of this current study, some limitations and possible confounders need to be considered. Factors like susceptibility to infection, multiple organ dysfunction, haemodialysis and mechanical ventilation were not taken into account. Also the time of sample collection needs to be contemplated on. All biomarker plasma levels entering our statistical analysis originate only from one baseline testing at the beginning of study inclusion. Further testing on biomarker levels in the days following inclusion (e.g. every day or every other day) and their consideration in statistical analysis might approve advantageous for more accurate results as infection markers like cytokines follow different patterns concerning concentration changes over the course of the response to infection (5,6).

In conclusion, our study rated PCT, suPAR, biotin, fibronectin and IL-8 as the most robust laboratory infection markers, for prediction of septicemia and 30-days mortality among SIRS patients. Larger studies are needed to evaluate the clinical diagnostic significance of these biomarkers in determination and prognosis of sepsis.

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