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Initial anergy of immune response in patients with severe sepsis and septic shock - implications for future treatment

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1. Introduction

1.1. Sepsis

1.1.1. Historical background

Sepsis and sepsis-associated conditions have long been discussed during the course of history. It was in 100 before Christ when Roman scholar and writer Marcus Terentius Varro first noted that “small creatures, invisible to the eye, fill the atmosphere, and breathed through the nose cause dangerous diseases” [1]. Even though technical possibilities at that time did not allow for specifying these observations any further, it was as far back as over 2000 years that people assumed a microbial, infectious genesis of the disease that we can only hypothesize might have been most probably sepsis. It was Sir William Osler (1849-1919) who observed that the patient appeared to die from the body’s response to the infection rather than from the pathogen itself [1]. With this finding, he identified the crux of sepsis over 100 years ago, which makes it the dangerous and difficult-to-treat disease it is up to the present day.

1.1.2. Definitions

The American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) held a Consensus Conference in August 1991, which came up with precise, universal definitions for the terms systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis, septic shock and multiple organ dysfunction syndrome (MODS), which had been non-existent until then [1, 2] (*Table 1*).

Ten years later, several North American and European intensive care societies determined to amend these definitions to facilitate their clinical use. But even though experts on the field agreed on the need to revise the ACCP/SCCM definitions referred to above, no superior alternatives could be identified [3], which made the original definitions overall still valid until the conduct of this study.

Table 1. Definition criteria for sepsis-associated conditions

Condition	Definition criteria
Systemic inflammatory response syndrome (SIRS)	Presence of two or more of the following conditions: • Body temperature < 36°C or > 38°C • Heart rate > 90 bpm • Respiratory rate > 20/min or $PaCO_2$ < 32 mmHg • WBC < 4000 cells/mm^3, > 12000 cells/mm^3 or > 10% immature forms
Sepsis	SIRS as response to an infection.
Severe sepsis	Sepsis associated with organ dysfunction, hypoperfusion or hypotension.
Septic shock	Sepsis with hypotension, despite adequate fluid resuscitation, along with the presence of perfusion abnormalities.
Multiple organ dysfunction syndrome (MODS)	Presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention.

Adapted from 1992 ACCP/SCCM Consensus Conference definitions [2].

In 2016, the third international consensus definitions for sepsis and septic shock (Sepsis-3) [4] were published, focusing hereby on organ dysfunction accompanying suspected or proven infection rather than the clinical SIRS criteria used before. According to the authors, identification of sepsis by the old criteria was of poor sensitivity and specificity.

It is stated that the inadequate focus on inflammation and the common model of a continuum from local infection to non-complicated sepsis, which is proceeding further to severe sepsis (characterized by organ failure) and subsequently septic shock, are out of date [4].

1.1.3. Epidemiology

Sepsis, and in particular severe sepsis and septic shock, are still associated with high fatality and rising incidence. In the United States (US), they are the leading cause for death in critically ill patients [5], accounting for about as many annual deaths as acute myocardial infarction [6]. While sepsis is associated with a mortality of about 10 – 20 %, in severe sepsis this number is more than doubled (20 – 50 %). In septic shock it increases further to 40 – 80 % [1]. In the late 20th century, the incidence of sepsis annually increased by 8.7 % in the US, peaking in a total incidence of 240.4 per 100,000 population in 2000. Even though the total in-hospital mortality decreased from 27.8 % to 17.9 % in the same period of time, the total number of deaths continued to rise due to the increase in incidence [7]. The incidence of severe sepsis in first world countries is reported to be approximately 50 – 100 per 100,000 population [8], thus being three to four times lower than the incidence of sepsis [7]. Both severe sepsis and septic shock have occurrence rates in intensive care units (ICUs) of about ten percent [6, 9], debiting the health care system an annual $16.7 billion in the US alone [6]. There is by far less data available concerning epidemiology of sepsis in third world countries, but since it is generally acknowledged that infectious diseases - which are the inevitable cause for sepsis - play an even greater role in developing countries, one can only presume that sepsis might be of similar or even greater importance in the third world [1].

1.1.4. Risk factors

An individual person's risk to develop sepsis is affected by a multitude of patient-specific factors and the most important one seems to be age. While sepsis incidence showed to be 0.2/1000 in children, it increased more than a hundredfold in patients over the age of 85 years (26.2/1000) [6]. But also certain ethnic groups displayed an elevated risk to develop sepsis, with relative risks of about 1.9 as compared to Caucasians [7]. Furthermore, the male sex seems to be a predisposing factor, with a relative risk of 1.28 as compared to females [7]. Apart from that, certain medical conditions significantly raise one's probability to contract sepsis, most of which seem to impair the immune system. Diseases like diabetes mellitus, cancer or an infection with the human immunodeficiency virus (HIV) should be named in the first place in that context [1].

1.1.5. Causative pathogens

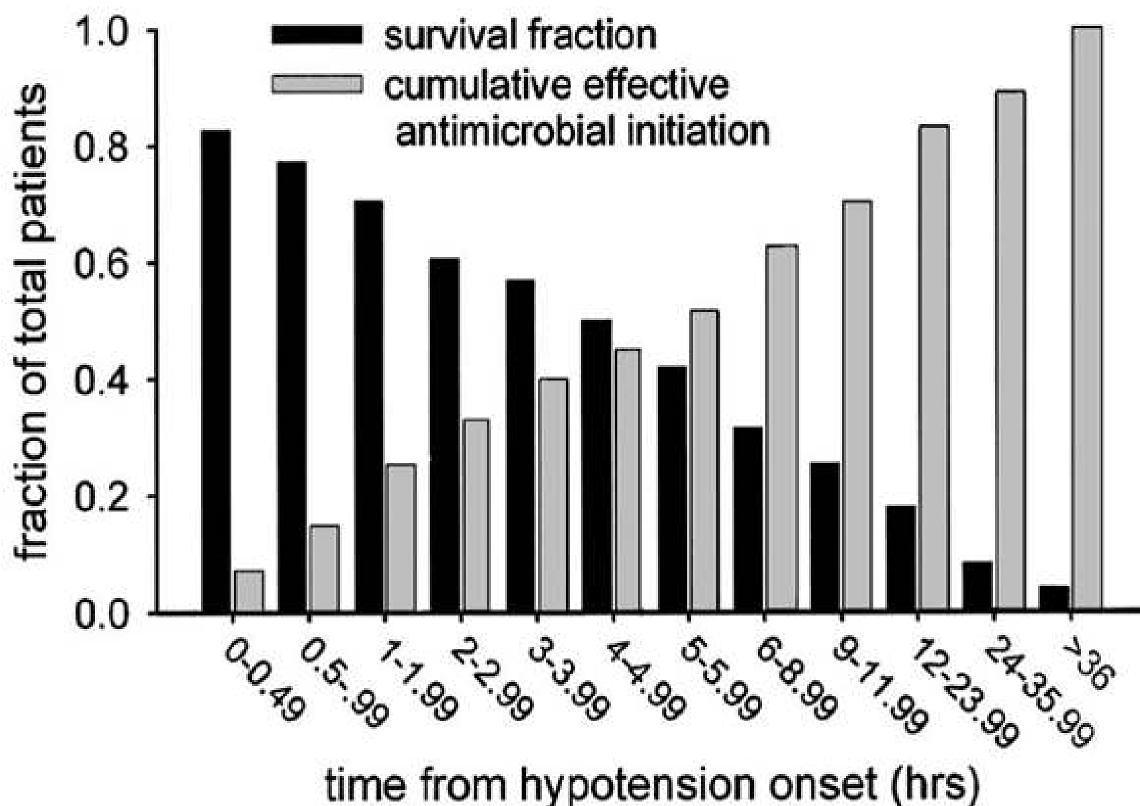
Since it was first assumed that lipopolysaccharide (LPS) played a crucial role in sepsis pathogenesis, gram-negative bacteria were originally blamed to be responsible for most of the sepsis cases, which was in fact confirmed by some studies [1, 10]. Recent studies, however, came up with the finding that gram-positive bacteria in these days are outnumbering gram-negative bacteria in originating sepsis, with 52.1 % of US cases in 2000 being gram-positive sepsis compared to 37.6 % of cases caused by gram-negative microorganisms [7]. Apart from these, viral and fungal organisms could be identified as possible causative organisms for sepsis [1], the latter of which accounted for 4.6 % of sepsis-cases, constituting an increase of 207 % in a period of 20 years [7].

1.1.6. Therapy

1.1.6.1. Causal therapy

Nowadays, the therapy of severe sepsis and septic shock can be roughly viewed as two-pronged: first, there is the crucial need for causal anti-infective therapy, which initially needs to cover a broad spectrum including all possible pathogens for the particular site of infection and should be administered as early as possible, directly after harvesting blood cultures [11]. As Kumar et al. could show in 2006, within the first six hours after the onset of hypotension, each hour of delay in the administration of antimicrobial therapy accounted for a mean decrease of 7.6 % in survival rate (Figure 1) [12]. If possible, there should be additional surgical or interventional infectious focus control in order to reduce the pathogen-load of the exposed body.

Figure 1. Dependency of survival on the early initiation of antimicrobial therapy



Cumulative effective antimicrobial initiation following onset of septic shock-associated hypotension and associated survival. The x-axis represents time (hrs) following first documentation of septic shock-associated hypotension. Black bars represent the fraction of patients surviving to hospital discharge for effective therapy initiated within the given time interval. The gray bars represent the cumulative fraction of patients having received effective antimicrobials at any given time point. From: Kumar et al., 2006 [12].

1.1.6.2. Supportive and adjunctive therapy

The second mandatory component of effective sepsis therapy is the support of the patient's vital functions, if necessary. Mechanical ventilation is often required, and in case of septic shock a hemodynamic therapy consisting of extensive fluid resuscitation and the administration of inotropic or vasopressor drugs is needed. In case of renal failure, the initiation of temporary renal replacement therapy (RRT) may be necessary. Patients may require prophylactic means (i.e. deep vein thrombosis prophylaxis, stress ulcer prophylaxis), the administration of blood products, hydrocortisone (HC), bicarbonate, analgesic or sedative agents, enteral or parenteral nutrition and blood glucose management via the adapted infusion of glucose and insulin in the critical phase of their disease. Moreover, a surgical approach may be required, depending on the individual case [11, 13].

1.2. The immune system

1.2.1. Physiology

Crucial to the understanding of sepsis pathogenesis is the knowledge of the human immune system. In general, it can be divided into an innate (nonspecific) and acquired (specific) part, although both systems are strongly interacting by enhancing or modulating each other's responses.

1.2.1.1. Innate immune system

The innate immune system is composed of a cellular as well as a humoral pathway, the first of which is predominantly represented by neutrophils, macrophages and natural killer cells, whilst the latter consists of antimicrobial substances, such as components of the complement system, which are released into the blood and interstitial fluids. In simplified terms, the innate immune system divides the world into the harmless "self" and the potentially dangerous "non-self", dependent on physical structure. One main clue for the decision whether to classify a certain antigen as self or non-self is the recognition of particular carbohydrates, lipids, proteins or deoxyribonucleic acid (DNA) structures, which frequently occur among microbial invaders. For instance, the Cluster of Differentiation (CD) 14 cell surface protein on macrophages serves the purpose of identifying LPS, which is a very common component of the gram-negative bacterial cell wall and thus indicative of bacterial infection when appearing in human bodily fluids [14].

1.2.1.2. Acquired immune system

Besides the nonspecific innate immune system, the body is equipped with the more specific acquired immune system. Similar to the innate immune system, the acquired immune system also relies on cellular as well as humoral components. It is mainly mediated by lymphocytes, which can be divided, depending on the site of their maturation (indicated in brackets hereafter), in B-lymphocytes (bone marrow), which are responsible for the humoral part by secreting immunoglobulins, and T-lymphocytes (thymus), which mediate the cellular response to pathogens. T cells can be further divided into cytotoxic CD8+ cells, which kill their target cells by secreting lytic substances or by inducing apoptosis via the Fas ligand pathway, and CD4+ helper T cells (TH). The latter can be even further divided into two different main types of effector cells, TH_1 and TH_2. While the main purpose of TH_1 cells has long been thought to be promoting cellular immune response by activating macrophages, natural killer cells and neutrophils, TH_2 cells were considered to be in charge of stimulating the B-cell mediated specific humoral immunity [15, 16]. Communication between immune cells for the most part is performed via the release of cytokines, which can be viewed as messengers between cells. Leukocytes can activate or inhibit their surrounding cells by releasing cytokines in a paracrine fashion, or even affect themselves by using cytokines in an autocrine fashion. When having a closer look at the signature cytokines of both TH_1 and TH_2 cells, it becomes apparent that besides affecting different effector cells and thus the different paths of the immune system, their main effect on the inflammatory response seems to be contrary. While TH_1 cells mainly produce

cytokines like interferon (IFN)-γ, interleukin (IL)-2 and tumor necrosis factor (TNF)-α, all of which recruit and activate inflammatory leukocytes and thus have pro-inflammatory effects, three of the most important cytokines typically released by TH_2 cells, namely IL-4, IL-10 and IL-13, exert anti-inflammatory effects [16].

1.2.1.3. Processes occurring during infections

In case of an infection, pathogens like bacteria, viruses or fungi enter a sterile site in the human body and are detected and phagocytosed by components of the innate immune system. Simultaneously, after recognizing particular structural patterns of the invading microorganisms, so-called pathogen-associated molecular patterns (PAMPs), the innate immune system alerts the host of the infection by secreting cytokines. These on the one hand increase the innate immune response by recruiting more leukocytes to the site of infection and, on the other hand, activate the adaptive immune system in a very complex way of interlinkage between the two parts of the immune system [17]. If the pathogen-load the body is exposed to can be handled by the host's initial immune response, the inflammatory process is regulated and terminated by - amongst others - TH_2 cells, and homeostasis of the body is gradually restored [16].

1.2.2. Pathophysiology of septic conditions

1.2.2.1. Systemic inflammatory response syndrom

In case of a persistent or particularly severe infection, the immune responses are potentiated by the activation of leukocytes by so-called damage-associated molecular patterns (DAMPs), which are molecular structures being released from damaged host tissue [18]. This process can advance into a vicious circle, with progressive inflammation entailing more tissue damage, and thus resulting in even more inflammation. Sepsis is present when the inflammatory process within the host increases to such a level that SIRS develops and the body reacts with systemic changes [17]. These can be neuroendocrine, hematopoietic, metabolic or hepatic changes [15]. If the infection persists and the body's reaction exacerbates, organ dysfunction, tissue hypoperfusion or hypotension may occur, marking the emergence of severe sepsis. Septic shock is present when hypotension rises to such a level that, despite adequate intravenous fluid resuscitation, the administration of inotropic or vasopressor agents is required to maintain an adequate mean arterial blood pressure (MAP) [2]. These general changes to the body are partly mediated by an overwhelming amount of cytokines often termed as "cytokine-storm", in which the cytokines not only affect surrounding cells in an autocrine and paracrine manner, but are released in such a quantity that they have an endocrine effect on all body tissues and contribute to the effects pointed out above [18]. Secondly, the exaggerated formation of molecules such as hydrogen peroxide, superoxide radical anion and hydroxyl radical - which are subsumed as reactive oxygen species (ROS) - is widely recognized to play a role in the genesis of the sepsis-associated sequelae named above, like cardiovascular insufficiency and tissue injury. These substances are mostly generated by macrophages and neutrophils [14, 19]. A third contributing factor seems to be the release of proteases and other antimicrobial peptides [17].

1.2.2.2. Compensatory and mixed anti-inflammatory response syndrome

Besides SIRS, which still is the hallmark sign of sepsis, another contrary condition in the progression of sepsis is described in the literature. It is associated with an inhibition of the immune system, resulting in a lack of response to pathogens, and goes by different names, such as compensatory anti-inflammatory response syndrome (CARS) or immune paralysis [18, 20, 21]. This condition causes a severe susceptibility to secondary infection and might be responsible for a significant number of deaths in the later phases of sepsis. There are different hypotheses concerning this immunosuppressive state. It is assumed that a shift from a TH_1-dominated initial immune response resulting in excessive inflammation and, subsequently, SIRS, to a TH_2-dominated anti-inflammatory state might contribute to the development of CARS [5]. Different works state that extensive lymphocyte apoptosis during sepsis progression seems to be, at least in part, responsible for the genesis of CARS [22]. Recent findings suggest that hyperinflammation and hypoinflammation are two concurrently developing processes in sepsis, terming it as mixed anti-inflammatory response syndrome (MARS) [20, 23]. There are numerous further theories concerning the pathophysiology of immunosuppression in sepsis, including impaired leukocyte recruitment and decreased cell surface protein expression [24]. After all, the exact pathophysiology of sepsis and the accompanying hyperinflammatory and immunosuppressive states are still poorly understood, despite great efforts in research on this topic. A better understanding of these conditions is needed to eventually make considerable therapeutic advances.

1.3. Goals of the work

To the present day, there have been numerous approaches on modulating the patient's immune response in sepsis, none of which has been promisingly successful in clinical trials until now [5]. The aim of this work was to investigate cellular leukocyte responsiveness of inpatients admitted to an ICU suffering from severe sepsis or septic shock.

The first main objective was to evaluate the patients' initial immune function, determined shortly after the onset of severe sepsis or septic shock, respectively, in comparison to a healthy control group. We hypothesized that there would be general immunosuppression in patients and intended to further quantify and distinguish this condition.

Secondly, we targeted to identify different parameters correlating with the level of immune dysfunction, such as demographic, biometric, clinical, immunological, and outcome-parameters.

Thirdly, dependent on the findings in the items named above, we aimed at setting on possible hypotheses for pathophysiologic processes occurring during sepsis, thus contributing to a better understanding of sepsis pathogenesis. This study might constitute a small step towards future diagnostic and therapeutic strategies, as currently no single biomarker is available for clinical use in the assessment of immune dysfunction [24].

2. Materials and methods

2.1. Study design

2.1.1. SISPCT

Patients were enrolled in our study as participants of a superordinate study designed as a prospective, randomized, multicentric clinical trial named “Placebo Controlled Trial of Sodium Selenite and Procalcitonin Guided Antimicrobial Therapy in Severe Sepsis” (SISPCT; clinicaltrials.gov identifier: NCT00832039 [25]) designated to enrol 1.180 patients in various German ICUs. Aim of this trial was to investigate the influence of sodium selenite infusions (randomized, double-blind) and Procalcitonin (PCT) guided causal therapy (randomized, open-label) on the survival and various secondary outcome measures of patients suffering from severe sepsis or septic shock. Patient recruitment for SISPCT was started in November 2009 and ended in March 2013 [25].

2.1.1.1. Inclusion criteria

In order to qualify for study enrolment, patients had to meet the criteria for either severe sepsis or septic shock according to the ACCP/SCCM definitions valid at the time of study conduct [2]. First, there had to be a clinically suspected or microbiologically proven infection in combination with two or more of the SIRS-criteria, indicating the presence of sepsis (*Table 1*). As opposed to the original ACCP/SCCM definitions as seen in *Table 1*, all interval boundaries (e.g. 38°C, 90 bpm, etc.) were included to fulfil the criteria. Moreover, the necessity of mechanical ventilation alone qualified to fulfil the respiratory criterion, besides an elevated respiratory rate and a decreased $PaCO_2$. In addition, in order to meet severe sepsis or septic shock definitions, one or more of the following had to be present: acute encephalopathy, thrombocytopenia, renal dysfunction, metabolic acidosis, arterial hypoxemia, arterial hypotension or septic shock (*Table 2).* Patients had to be enrolled within a time frame of 24 hours after onset of severe sepsis or septic shock.

Table 2. SISPCT criteria for severe sepsis and septic shock

Condition	Organ dysfunction	Definition criteria
Severe sepsis	Acute encephalopathy	Reduced vigilance, anxiety, disorientation, without interference by psychotropic agents.
	Thrombocytopenia	Thrombocytes ≤ 100,000/µl or decrease in thrombocytes > 30% within 24 hours without being caused by blood loss.
	Renal dysfunction	Urinary output ≤ 0.5 ml/kg/h despite sufficient fluid substitution and/or a rise of serum creatinine ≥ 2 x above reference range.
	Metabolic acidosis	Base excess ≤ -5 mmol/l and/or plasma lactate concentration ≥ 1.5 x above reference range.
	Arterial hypoxemia	PaO_2 < 10 kPa (75 mmHg) without oxygen administration or PaO_2/FiO_2 ≤ 33 kPa (250 mmHg) under oxygen administration without presence of a cardiac or pulmonary disease accountable for hypoxemia.
	Arterial hypotension	Systolic blood pressure ≤ 90 mmHg or MAP ≤ 70 mmHg for at least 1 hour despite sufficient fluid administration under absence of other causes for circulatory shock.
Septic shock		Systolic blood pressure ≤ 90 mmHg or MAP ≤ 70 mmHg for at least 2 hours despite sufficient fluid administration or necessity of vasopressor agents to establish a systolic blood pressure ≥ 90 mmHg or MAP ≥ 70 mmHg under absence of other causes for circulatory shock.

As part of the SISPCT inclusion criteria, one or more of these criteria had to be present in addition to the presence of sepsis to allow for patient enrolment. PaO_2: Arterial partial pressure of oxygen, kPa: Kilopascal, mmHg: Millimetres of mercury, FiO_2: Fraction of inspired oxygen, MAP: Mean arterial pressure.

2.1.1.2. Exclusion criteria

Patients who met one or more of the following exclusion criteria at screening could not be enrolled: pregnancy, breastfeeding period, selenium intoxication, an infection in which guidelines suggest a long-term antimicrobial therapy, therapy limitation or termination, infaust prognosis, severely immunologically compromised patients with $CD4^+$ counts < $200/mm^3$ or neutrophils < $500/mm^3$ or pharmacological immunosuppression in status post solid organ transplantation, participation in another clinical trial within the last 30 days, current participation in another research project, earlier participation in SISPCT or a personal relation to the principal investigator.

2.1.1.3. Informed consent procedure

After positive patient screening suggesting patient enrolment, either an informed consent signed by the patient or, in case of impaired legal competence at that time - e.g. due to acute encephalopathy or analgesic sedation - a surrogate confirmation had to be present in order to be able to enrol the patient. The surrogate confirmation could either be the signed consent of a patient's medical representative or, if none was available, a written declaration of a medical consultant from a different medical subject allowing patient enrolment. In case of patients having been enrolled based on a medical consultant's declaration, an informed patient's or medical representative's consent or refusal had to be obtained within a few days after study enrolment. In case of subsequent refusal, the patient concerned was excluded from the study immediately and no further follow-up was performed.

2.1.1.4. SISPCT study arms

Directly after study enrolment patients received a bolus, which was administered over the course of 20 minutes via a central venous catheter. It consisted of either 1000 µg of selenium, dissolved as sodium selenite pentahydrate (selenase® T pro injectione, biosyn Arzneimittel GmbH, Fellbach, Germany) in aqueous 0,9 % sodium chloride solution (50 ml total volume) or the same amount of placebo in the form of 50 ml of aqueous 0,9 % sodium chloride solution. Subsequently, patients received a continuous infusion of sodium selenite pentahydrate, dissolved as described above, at a rate of 1000 µg of selenium per 24 hours, or the same volume of placebo, respectively. Assignment to either of these two study arms took place in a randomized, double-blinded fashion. The continuous infusion was administered for the duration of the patient's stay on ICU, but maximum for 21 days.

Independently from this, all patients were assigned to either the PCT guided causal therapy arm or the control arm. In the PCT guided arm, causal therapy, i.e. antimicrobial therapy, interventions for focus control and diagnostic measures were supposed to be directed according to a certain algorithm based on plasma PCT levels on certain days after study enrolment. For the control group, no plasma PCT levels were determined and no therapeutic suggestions were made. Assignment to either of these two arms was performed in a randomized, open-label fashion.

2.1.1.5. Study procedures

During the length of ICU stay, but maximum for 21 days, clinical, laboratory and therapeutic parameters were recorded in detail. Additional follow-ups were scheduled for day 28 and day 90 after study enrolment. During stay on ICU, blood samples were taken on Days 0, 1, 2, 3, 4, 5, 6, 7, 10, 14 and 21 for later analysis. Naturally, all study interventions and inquiries were implemented in addition to the usual critical care treatment necessary.

2.1.2. Immune function substudy

From June 2011 through February 2013 we recruited a total of 76 patients for the additional assessment of their immune function, besides their regular participation in SISPCT (Amendment of ethical approval: Eudra-CT-Nr. 2007-004333-42). All of these patients were recruited in one particular of SISPCT's trial sites: Ludwig-Maximilian University of Munich, Klinikum der Universität München, Klinikum Großhadern, Department of Anaesthesiology, on the ICUs “H2”, “H3b” and “I3”, respectively. Inclusion and exclusion criteria were identical to those of SISPCT, whereas the different SISPCT study arms were not considered dissociatedly in our study due to the small sample size and the blood withdrawal taking place before administration of the study drugs. In the following, “immune study” shall refer to the tests on immune function, whereas “SISPCT” represents the multicentre trial described above. Complementary to the patient population, we recruited 11 age- and sex-matched healthy volunteers (HV) as a control group between September 2012 and December 2012 to participate in the study after giving signed informed consent. In regard to gender distribution, age and body mass index, the control group and patient collective did not differ significantly (*Table 3*).

Table 3. Demographic and biometric characteristics of patients versus control group

Variable	Patient Population	Control group
N	76	11
Female, n (%)	35 (46 %)	3 (27 %)
Age (years) [IQR]	65 [50 – 73]	54 [48 – 64]
Body mass index (kg/m^2) [IQR]	26 [23 – 32]	25 [23 – 29]

No significant group differences were detected regarding gender distribution (Fisher's exact test, p = 0,335), age (Pearson's chi-squared test, p = 0,249) and body mass index (Pearson's chi-squared test, p = 0,248).

2.2. Laboratory methods

2.2.1. Blood sample obtainment

Subsequent to patient enrolment (Day 0) or, in case of the control group, at a time of subjective physical well-being, 9 ml of blood was withdrawn into a lithium-heparinized tube (S-Monovette® 9 ml, Lithium-Heparin, 92x16 mm, Sarstedt AG & Co., Nümbrecht, Germany). For patient blood sample obtainment, a pre-existent arterial catheter or - if none was available at the time - a central venous access was used. In case of the control group, single peripheral vein puncture was performed.

2.2.2. Whole blood stimulation

Subsequently, 400 µl of lithium-heparinized whole blood was transferred under aseptic conditions into each tube prefilled with an equal volume (400 µl) of DMEM (Dulbecco´s Modified Eagle's Medium Nutrient Mixture F-12 HAM, Sigma-Aldrich, Steinheim, Germany) and the different stimulants (800 µl total assay volume). The assay tubes contained DMEM only or DMEM and either a bacterial antigen mixture (Bacteria) containing diphteria-, tetanus- and pertussis-toxoid (all 3 combined in 1% Boostrix®, GlaxoSmithKline, Munich, Germany), or fungal antigen mixture (Fungi) containing candida-lysate (10 µg/ml, Allergopharma, Reinbeck, Germany) and trichophyton-lysate (10 µg/ml, Allergopharma, Reinbeck, Germany) or Pokeweed mitogen (PWM) (5 µg/ml, Sigma-Aldrich, Steinheim, Germany) as positive control. For specific lymphocyte stimulation, we chose the bacterial antigens named above, because they constitute some of the antigens used in standard vaccinations recommended by the public authorities [26]. The fungal species employed are characterized by an ubiquitous occurrence in the environment. It can be assumed that almost all patients and HVs had been exposed to the chosen antigens prior to study participation and thus were equipped with specific memory cells, enabling them to exert a prompt specific immune response. PWM acts as a strong "polyclonal" activator, inducing mitosis in T as well as B lymphocytes in a maximal but non-receptor specific way [27, 28].

Additionally, there were three more stimulation solutions kindly provided by the National Aeronautics and Space Administration (NASA) (Washington, District of Columbia, USA), which were included at a later time, beginning with the 19th study patient. In case of these reagents, 150 µl of whole blood was transferred under aseptic conditions into each tube prefilled with 1 ml of Roswell Park Memorial Institute medium (RPMI) containing LPS (10 µg/ml, Sigma-Aldrich, St. Louis, Missouri, USA), phorbol 12-myristate 13-acetate and ionomycin (PMA, 10 ng/ml PMA and 2 µg/ml ionomycin, both from Sigma-Aldrich, St. Louis, Missouri, USA) or human T-activator CD3/CD28 (CD3/28; 0.125 µg/ml anti-CD3 and 0.25 µg/ml anti-CD28; both from Becton Dickinson, Franklin Lakes, New Jersey, USA). LPS functions as an activator of cells of the innate immune system via the CD14 cell surface receptor and Toll like receptor (TLR) 4 signaling cascade [29]. PMA is a potent biologic stimulus [30] whose effect is increased when applied in conjunction with ionomycin. It serves as an unspecific activator of Protein kinase C (PKC) [31] and thus affects multiple cell types. Besides serving as a mitogen for T lymphocytes [32], it seems to have pro-inflammatory effects [30] and thus causes morphologic changes as well as an increased degranulation, ROS-generation, and phagocytosis in innate immune cells [33-35]. CD3/28 is a solution intended for

physiological activation of the CD3 antigen, which constitutes the human T cell receptor, and CD28, which is a receptor exerting costimulatory effects on the human T cell receptor [36]. These two receptors seem to occur only in T cells [37], which makes CD3/28 a reagent stimulating exclusively T lymphocytes.

2.2.3. Sample processing and analysis

The assay tubes were incubated altogether for 48h at 37°C. After 48 hours, the following were transferred into Eppendorf tubes and immediately frozen at -80°C for future cytokine analyses: 150 µl of supernatant plasma in case of tubes containing just DMEM, bacterial / fungal antigens or PWM, or 200 µl of supernatant plasma in case of tubes containing LPS, PMA and CD3/28, respectively. By rarefying these small supernatant volumes significant dilution effects could be minimized. Frozen supernatants were measured in a blinded fashion after thawing.

In case of tubes containing just DMEM, bacterial / fungal antigens or PWM the concentrations of the prototypic TH_1 cytokines IL-2, IFN-γ and TNF-α were analyzed by Luminex xMAP® technology (Bioplex®) with commercially available reagents from BioRad-Laboratories Inc. (California, USA) according to the manufacturer's guidelines [38]. Data was analyzed using Bioplex®-Software.

Cytometric bead array (CBA) assessments were performed on supernatants from the tubes containing RPMI and LPS, PMA or CD3/28. CBA assays simultaneously measure multiple analytes using antibody-coated bead populations with unique fluorescence intensities. For the CD3/28 and PMA cultures, a TH_1/ TH_2 CBA assessment (Becton Dickinson, Human Th1/Th2 Cytokine Kit, Catalogue Number 550749) that analyzed secreted IFN-γ, TNF-α, IL-10, IL-5, IL-4 and IL-2 was performed. For LPS cultures, an inflammatory CBA assay (Becton Dickinson, Human Inflammatory Cytokines Kit, Catalogue Number 551811) was performed, which assessed secreted TNF-α, IL-10, IL-6, IL-1β, IL-8, and IL-12. All CBA assays were performed according to the manufacturer's instructions. Samples were batch-analyzed and a Beckman Coulter flow cytometer was configured to resolve all bead populations. Data were recorded as mean fluorescence intensity (MFI) and subsequently converted to pg/ml concentrations by plotting the subject MFI data against the MFI values established from a standard curve, according to Crucian et al. [39].

2.3. Electronic data processing

2.3.1. Data recording

Data recording was performed in an anonymized way using IBM SPSS® Statistics V21 (IBM Corp., New York City, New York, USA). We defined the time span of 24 hours before study enrolment as "Baseline" and the time from study enrolment to 7 a.m. the next day as "Day 0". The following days were consecutively numbered, with each day beginning and ending at 7 a.m. In addition to the acquisition of demographic, biometric, anamnestic, clinical and laboratory data and therapeutic procedures in each individual patient, the common severity of disease classification systems Simplified Acute Physiology Score (SAPS II) [40] and Acute Physiology And Chronic Health Evaluation II (APACHE II) [41] were calculated for the Baseline period. Cytokine data were included in the database after determination. In case of cytokine concentration values below the lower detection limit, we used the value of the lower detection limit in order to be able to perform statistic calculations.

2.3.2. Statistical analysis and plotting

Data was statistically analyzed and graphically plotted using IBM SPSS® Statistics as well as SigmaPlot 12.5 (Systat Software Inc., San Jose, California, USA).

In order not to distort raw data, no outlier analysis was performed. After testing for normal distribution using One-Sample Kolmogorov-Smirnov test or Shapiro-Wilk test, data was analyzed using Student's T-test, Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks or Mann-Whitney Rank Sum Test, dependent on presence of normal distribution. Correlation analyses were performed using Pearson's correlation coefficient or Spearman rank-order correlation coefficient, as appropriate. Normally distributed continuous data is given as mean ± standard deviation (SD), not normally distributed data is given as median [interquartile range (IQR)]. All p-values were calculated in a two-sided manner and statistical significance was set at an α-value of 0,05.

For better comparability of not normally distributed data, variable values were divided into specific groups (indicated in the individual charts) or four quartile groups, with the 25th, 50th and 75th percentile representing the cutoff values for group allocation. In boxplots, boxes show the median and IQR, whiskers represent the 10th and 90th percentile.

3. Results

3.1. Study population

76 Patients were included in the study, seven of which later refused consent or were lost to follow up within the follow up period of 90 days. All patients experienced onset of severe sepsis (8 %) or septic shock (92 %) within 24 hours prior to study enrolment (*Table 4*).

Table 4. Sepsis and admission type characteristics

	Total n = 76		**Admission type**		
	Severe sepsis	**Septic shock**	**Medical**	**Non-scheduled surgical**	**Scheduled surgical**
N (%)	6 (8 %)	70 (92 %)	41 (54 %)	27 (36 %)	8 (11 %)

96 % of patients were on antimicrobial therapy at the time of study enrolment. Most frequent focus localization was the respiratory tract (53 %), followed by the abdomen (19 %) and urinary tract (7 %). Detection of pathogens causing sepsis could be accomplished in 51 % of cases, 53 % of which were gram-negative bacterial, as compared with 38 % of gram-positive bacterial and 8 % of viral sepsis (*Table 5*). Within the study population, median Baseline SAPS II was 67, APACHE II averaged 27 and - for the main part due to frequent analgosedation - patients attained a median of 3 on the Glasgow Coma Scale (GCS). Mechanical ventilation was performed in 83 % of patients. 95 % of patients were on inotropic or vasopressor agents at the time of study enrolment, namely norepinephrine solely or in

Table 5. Microbiological and infection characteristics

Variable	**N (%)**
Microbiologically proven infection	39 (51 %)
Gram-positive bacterial	15 (38 %)[a]
Gram-negative bacterial	21 (54 %)[a]
Viral	3 (8 %)[a]
Focus of infection	
Respiratory tract	40 (53 %)
Abdomen	14 (19 %)
Urinary tract	5 (7 %)
Other	17 (22 %)
Baseline antiinfective therapy[b]	73 (96 %)

[a] Percentages given with reference to the number of microbiologically proven infections. [b] Number of patients who received antimicrobial substances within 24 hours prior to study enrolment.

combination with vasopressin (31 %), epinephrine (27 %) and/or dobutamine (7 %) (*Table 6).* Median length of ICU stay was 11 days. 35 % of patients required renal replacement therapy while on ICU and 12 % of patients deceased during ICU stay. 90-day mortality was 20 % (*Table 7*). On average, patients presented a considerable rise in infection parameters, a hematopoietic left shift, relative lymphopenia and relative granulocytosis, accompanied by moderate anemia and renal impairment. For a detailed cross section on Baseline laboratory values see *Table 8.*

Table 6. Baseline medical status	
SAPS II	67 [52 – 78]
APACHE II	27 ± 8
Glasgow coma score	3 [3 – 14]
Norepinephrine administered	70 (95 %)[a,b]
Maximum norepinephrine dose (mg/h)	1,9 [0,9 – 3,0]
Vasopressin administered	23 (31 %)[a]
Epinephrine administered	20 (27 %)[a]
Dobutamine administered	5 (7 %)[a]
Mechanical ventilation	63 (83 %)
Non-invasive	10 (13 %)
Invasive	53 (70 %)
Minimum PaO_2/FiO_2	118 [76 – 201]
Minimum arterial pH	7,29 ± 0,11

Characteristics for the patient population's medical status within 24 hours prior to study enrolment. Values are given as mean ± SD, median [IQR] or n (%). [a] Two missing values. Total n = 74. [b] Two patients with severe sepsis received norepinephrine status post non-scheduled abdominal surgery, but did not qualify for septic shock. SAPS II: Simplified Acute Physiology Score; APACHE II: Acute Physiology and Chronic Health Evaluation; PaO_2: arterial partial pressure of oxygen; FiO_2: fraction of inspired oxygen.

Table 7. Outcome characteristics	
Renal replacement therapy[a], n (%)	26 (35 %)
Length of stay on ICU (days) [IQR]	11 [5 – 21]
ICU mortality, n (%)	8 (12 %)
90-day mortality, n (%)	14 (20 %)

[a] Initiation of renal replacement therapy during stay on ICU.

Table 8. Baseline laboratory values

Category	Variable (unit)	Value	n
Differential blood count[a]	Hemoglobin (g/dl)	10.1 [9,1 – 12,1]	65
	Hematocrit (%)	30,0 [27,2 – 33,2]	51
	Platelets (G/l)	192 ± 98	68
	White blood count (G/l)	14.6 ± 10.6	65
	Segmented neutrophils (%)	85 [72 – 91]	18
	Banded neutrophils (%)	13.8 ± 10.6	8
	Monocytes (%)	5.9 ± 3.6	18
	Lymphocytes (%)	6,5 [3,0 – 9,0]	20
Clinical chemistry[b]	Sodium (mmol/l)	140 ± 5	71
	Potassium (mmol/l)	4,2 ± 0,8	69
	Glucose (mg/dl)	151 ± 55	69
	Creatinine (mg/dl)	1,4 [1,0 – 2,4]	68
	Total bilirubin (mg/dl)	1,3 [0,9 – 2,2]	67
	GOT/ASAT (U/l)	51 [27 – 99]	62
	GPT/ALAT (U/l)	32 [20 – 57]	66
	Lipase (U/l)	22 [7 – 44]	50
	C-reactive protein (mg/dl)	17,9 [10,7 – 27,8]	68
	Interleukin 6 (pg/ml)	1248 [252 – 4673]	62
	Procalcitonin (ng/ml)	6,6 [1,9 – 21,3]	51
Coagulation[c]	INR	1,2 [1,1 – 1,5]	73
	aPTT (sec)	30 [26 – 38]	72
	Fibrinogen (mg/dl)	563 ± 230	58

Values were determined from [a] whole blood, [b] blood serum or [c] blood plasma. Dependent on the presence of normal distribution, values are given either as mean ± SD or as median [IQR], respectively. GOT: glutamic oxaloacetic transaminase; ASAT: aspartat aminotransferase; GPT: glutamate-pyruvate transaminase; ALAT: alanine aminotransferase; INR: International normalized ratio; aPTT: activated partial thromboplastin time.

3.2. SISPCT

Regarding the SISPCT endpoints "selenium vs placebo" as well as "PCT guided anti-infectious therapy vs no PCT guidance", neither our dataset (data not shown) nor the SISPCT trial showed any significant differences in 28-day mortality in either of the study arms. PCT guidance resulted in a reduction of antimicrobial exposure - which served as a secondary endpoint - of 4,5 % per 1000 patient years, however. Results of the SISPCT trial were published in the Journal of the American Medical Association (JAMA) Internal Medicine in 07/2016 [42].

With regard to the cytokine release data acquired at Baseline, before implementation of the study interventions, no significant differences regarding the study arms could be detected (data not shown). The distribution of the patients in our database to the four study arms was about equal, lacking statistical significant divergence (selenium, PCT guidance n = 20, 26,3 %; selenium, no PCT guidance n = 20, 26,3 %; placebo, PCT guidance n = 18, 23,7 %; placebo, no PCT guidance n = 18, 23,7 %; total n = 76). Due to the small sample size in our database and the lack of relevant significant effects of both aspects examined in the SISPCT intervention study, we did not take the different study arms into consideration in our further analyses.

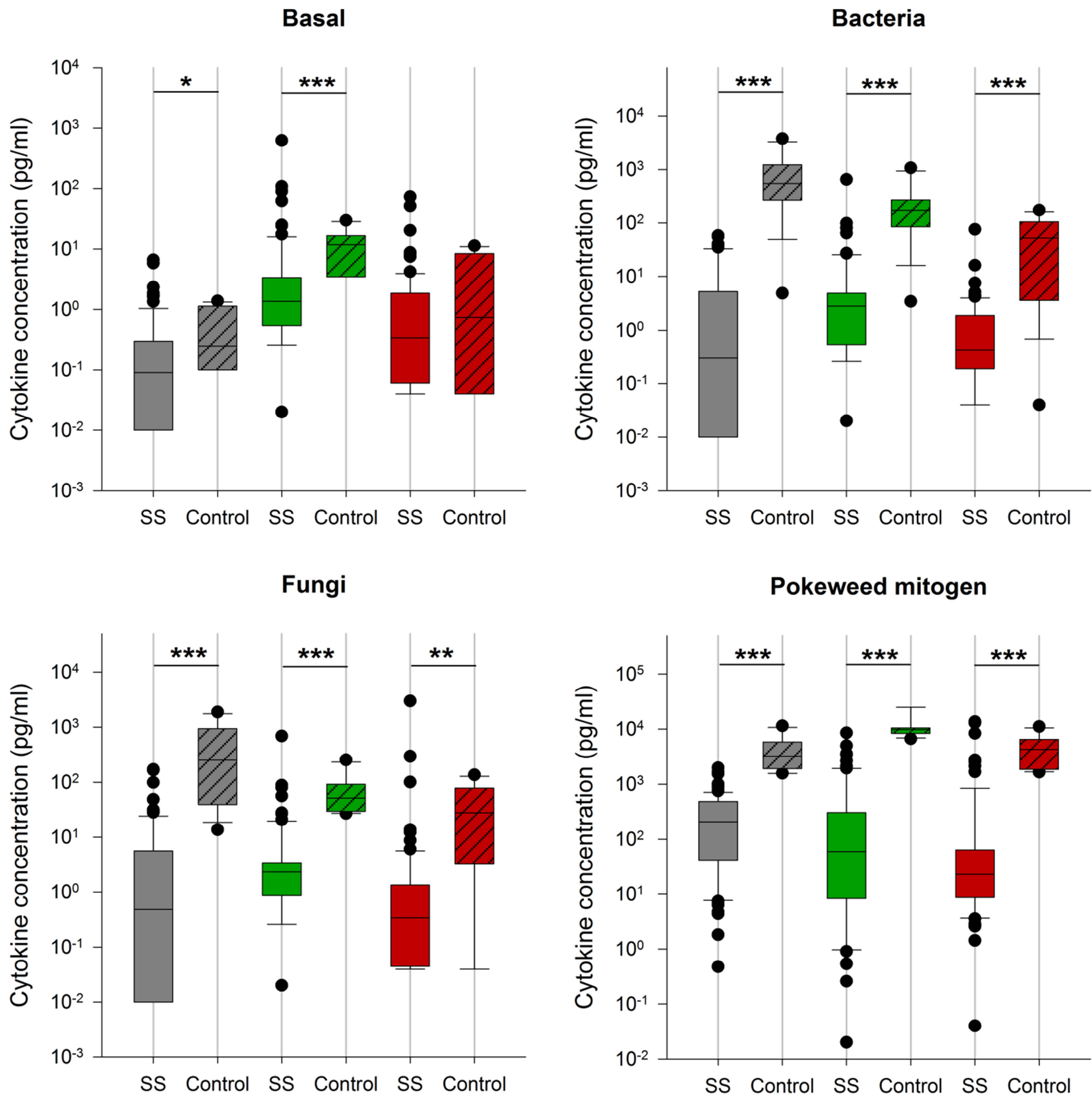
3.3. Initial immune function

All data used in this chapter refers to the situation at study enrolment and incorporates only values gathered in the defined study periods Baseline or Day 0, respectively.

3.3.1. Patients versus control group

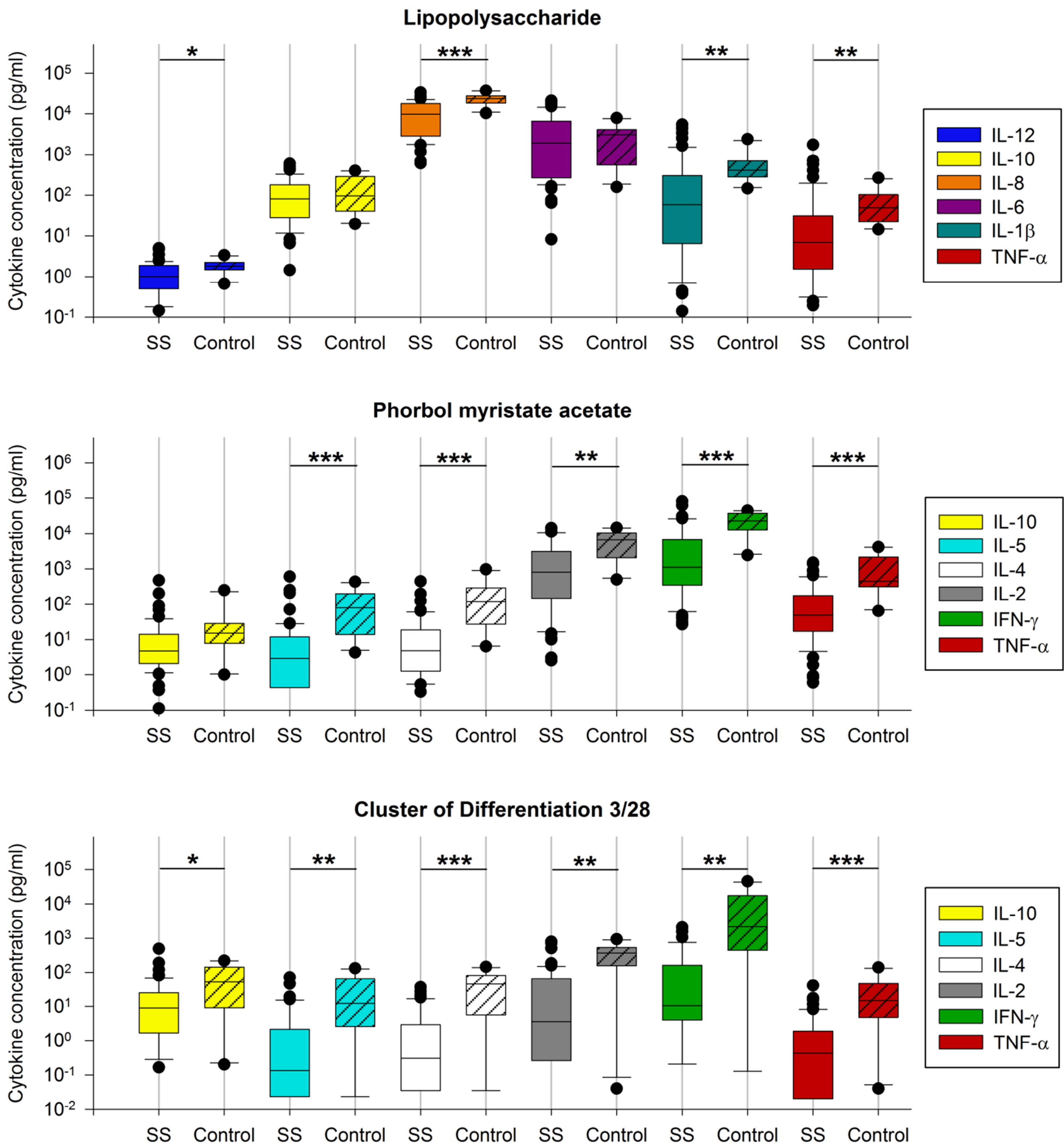
In comparison to the patients suffering from severe sepsis or septic shock (SS), a control group of 11 persons matching the patient population in age, sex ratio and BMI (as shown in *Table 3*) were included in the study. By assessing concentrations of different cytokines in the supernatants of whole blood incubated with various stimulants, their cellular immune function was compared to the patients' immune function at study enrolment. Results are displayed in *Figure 2* and *Figure 3*.

Figure 2. Patients versus control group in unstimulated assay and after stimulation with recall antigens and PWM



Interleukin 2, Interferon γ, :Tumor necrosis factor α, PWM: Pokeweed mitogen, SS: severe sepsis/septic shock patients, Basal: unstimulated test assay, Bacteria: bacterial antigen mixture, Fungi: fungal antigen mixture. Blood samples were taken subsequent to study enrolment (SS) or at a time of subjective physical well-being (control group), respectively. Statistically significant differences (Mann-Whitney Rank Sum Test) are indicated as follows: * $p < 0{,}05$, ** $p < 0{,}01$, *** $p < 0{,}001$.

Figure 3. Patients versus control group after stimulation with LPS, PMA and CD3/28



LPS: Lipopolysaccharide, PMA: Phorbol myristate acetate, CD3/28: Cluster of Differentiation 3/28, SS: severe sepsis/septic shock patients, IL: Interleukin, IFN: Interferon, TNF: Tumor necrosis factor. Blood samples were taken subsequent to study enrolment (SS) or at a time of subjective physical well-being (control group), respectively. Statistically significant differences (Mann-Whitney Rank Sum Test) are indicated as follows: * $p < 0{,}05$, ** $p < 0{,}01$, *** $p < 0{,}001$.

First of all, it is notable that in most cases (26 out of 30), significantly lower cytokine concentrations were found in the patient collective as compared to the healthy control group. Differences were in fact highly significant in the majority of cases ($p < 0,001$ in 16 and $p < 0,01$ in 7 out of 30 tested assays). These results indicate a severe, general cellular immune dysfunction which is already present shortly after the manifestation of severe sepsis or septic shock, respectively, and which doesn't seem to be restricted to specific cell types, since stimulants and measured cytokines of all types are affected. Even in the unstimulated assay, the control group showed a higher cytokine secretion than the patients, which was statistically significant for IL-2 and IFN-γ (*Figure 2*).

In detailed consideration of the results, it is remarkable that the assay stimulated with LPS, which fully addresses the innate immune system, showed the least significant results. Consistent with this, assays relying on a specific immune response were particularly affected by the suppression of cytokine release. After stimulation with bacterial or fungal antigen mixture, the release of the pro-inflammatory cytokines IL-2 and IFN-γ - which is predominantly mediated by T-lymphocytes [43] and thus dependent on the specific immune system - showed to be on a very low level compared to the control group (*Figure 2*, altogether $p < 0,001$).

In case of IL-10, which represents a prototypic cytokine exerting anti-inflammatory actions, statistically significant group differences only occur in the CD3/28 assay, but on a rather low significance level ($p < 0,05$). In the LPS and PMA assays, groups do not differ on a statistically significant level regarding IL-10 release (*Figure 3*). It is noteworthy, however, that patients did not exceed the control group in release of anti-inflammatory cytokines in any of the assays tested.

There is, however, a strong variability in the individual immune response, which occurs not only in the assays requiring prior antigen exposure and thus a specific immune response (namely the Bacteria and Fungi assays), but also is present in the assays using unspecific stimulants. This variability reaches dimensions of more than 10^3 under physiologic conditions (control group) and exceeds 10^5 in the patient collective (*Figure 2, Figure 3*). As a result, in some assays (i.e. Basal, LPS, PMA, cf. *Figure 2* and *Figure 3*) single patients exceed the control group in their cytokine response, whereas the majority of the patient collective undercuts the control group's values in all assays.

3.3.2. Subcohort analyses

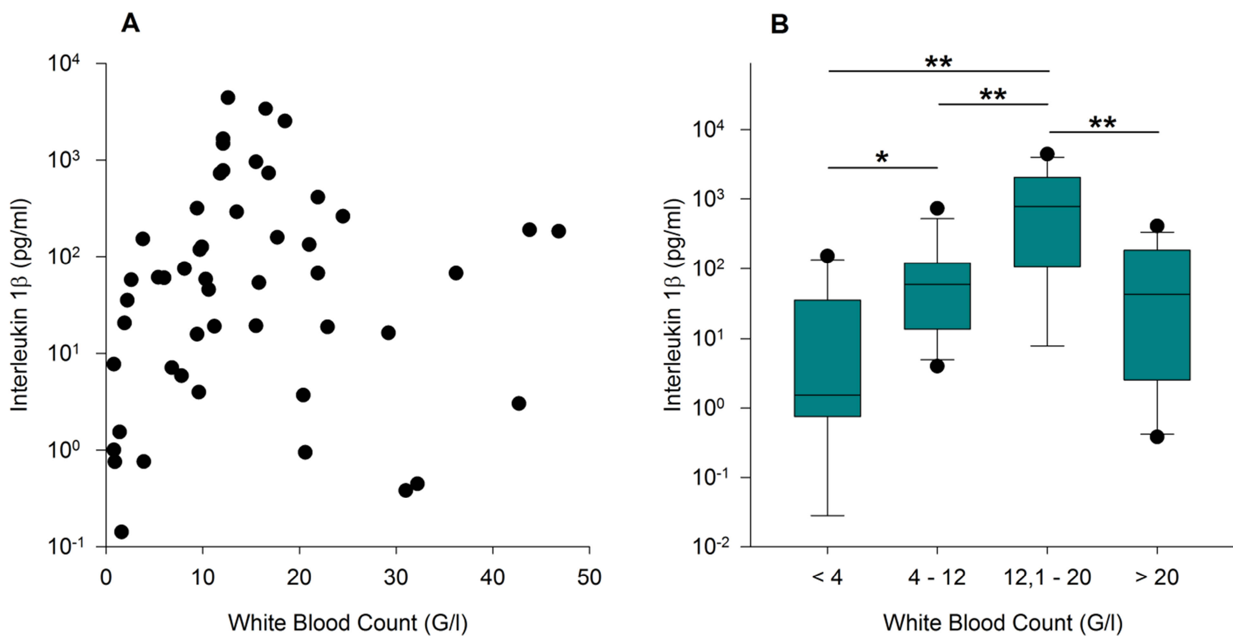
Examining the data for inter-patient variations in cytokine release, we made one general finding: in assays mostly addressing specific immunity (Bacteria, Fungi, CD3/28), significant discrimination between patients was not possible. This was due to the fact that, in these assays, a considerable fraction of the patients showed cytokine concentrations below the lower detection limit, which made a detailed analysis impossible (data not shown). This issue also affected the assays completely or partly relying on innate immunity (PWM, PMA1, LPS), but to a lower degree, which made the detection of correlations possible in these assays. In order to maintain consistency and comparability between different graphs and calculations, we restricted our considerations for the most part to two read-outs: the concentrations of IL-1β in LPS-stimulated whole blood and of TNF-α in PWM-stimulated whole blood, respectively.

3.3.2.1. White blood count

On the one hand, leukocytes are the cells that are responsible for releasing cytokines, so one could possibly assume that the WBC might show a positive correlation with cytokine release. But on the other hand, however, the WBC is an important inflammation marker: both ends of the spectrum, leukopenia as well as leukocytosis, can indicate inflammatory processes and thus both conditions represent one of the four SIRS criteria [2] (cf. *Table 1*). After calculating Spearman's rank correlation coefficients for WBC versus cytokine release in Day 0 stimulated whole blood, correlations significant on the .95 significance level could only be found in three out of 30 tested read-outs in a two-tailed test. In two out of the three cases, a negative correlation was found, while the remaining case showed a positive correlation (data not shown). This indicates that, considering the entirety of results, no stringent linear correlation is present.

Figure 4.A shows a scatterplot for patients' WBC versus IL-1β release after stimulation with LPS at day 0. It suggests a non-linear correlation with leukopenic and severely leukocytotic patients showing the faintest IL-1β release. *Figure 4.B* shows the corresponding boxplot after allocation of patients to four groups (leukopenia, normal leukocytes, moderate leukocytosis, severe leukocytosis). It shows that the group of patients with moderate leukocytosis (leukocytes 12,1 – 20 G/l) had the best immune response, whereas the leukopenic as well as the severely leukocytotic group showed a considerably impaired cytokine release.

Figure 4. Relationship between white blood count and IL-1β release after stimulation with LPS



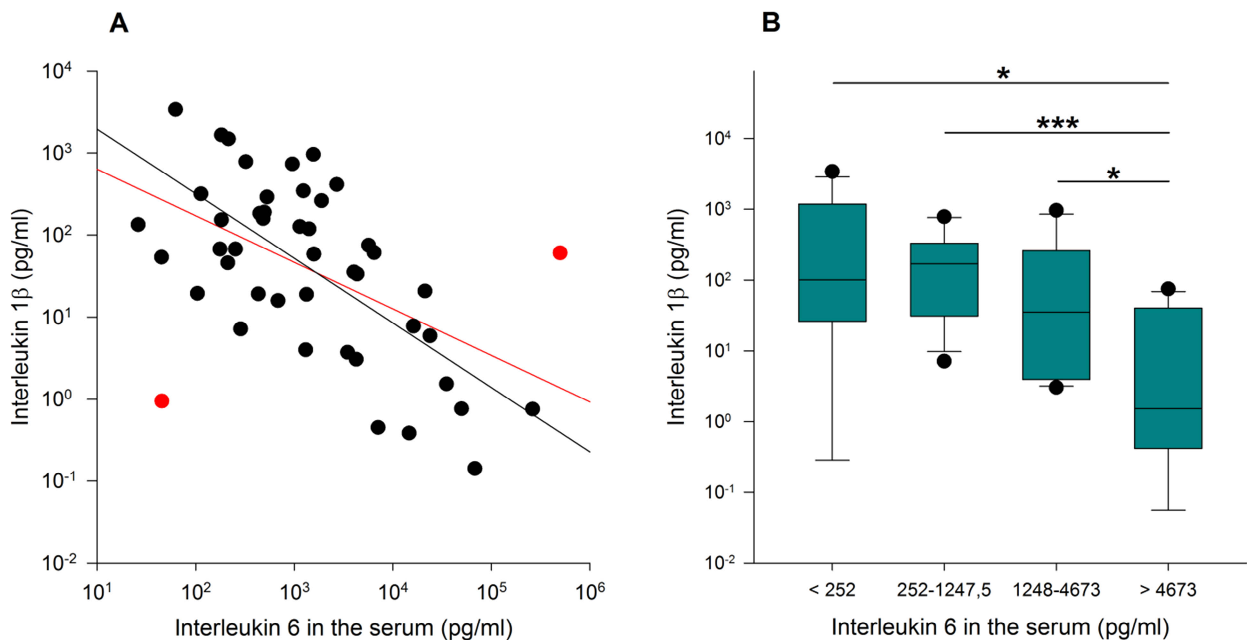
A) Scatterplot. B) Boxplot for four groups of patients in leukopenia (WBC < 4 G/l), with normal WBC (4 - 12 G/l), moderate leukocytosis (WBC 12,1 - 20 G/l) and severe leukocytosis (WBC > 20 G/l). Statistically significant differences (Mann-Whitney Rank Sum Test) are indicated as follows: * p < 0,05, ** p < 0,01.

3.3.2.2. Interleukin 6 serum level

Interleukin 6 (IL-6) serves as a reliable inflammatory marker in modern critical care medicine and is characterized by a fast dynamic, with a biological half-life period of less than 6 hours [44]. It thus represents an early indicator of inflammatory processes.

Figure 5.A shows a scatterplot for Baseline IL-6 determined in the blood serum versus IL-1β release after stimulation with LPS. A negative correlation becomes evident. For better statistical assessment of the underlying correlation, quartile groups were formed for IL-6 baseline values (*Figure 5.B*), proving the statistical significance of the formerly mentioned negative correlation.

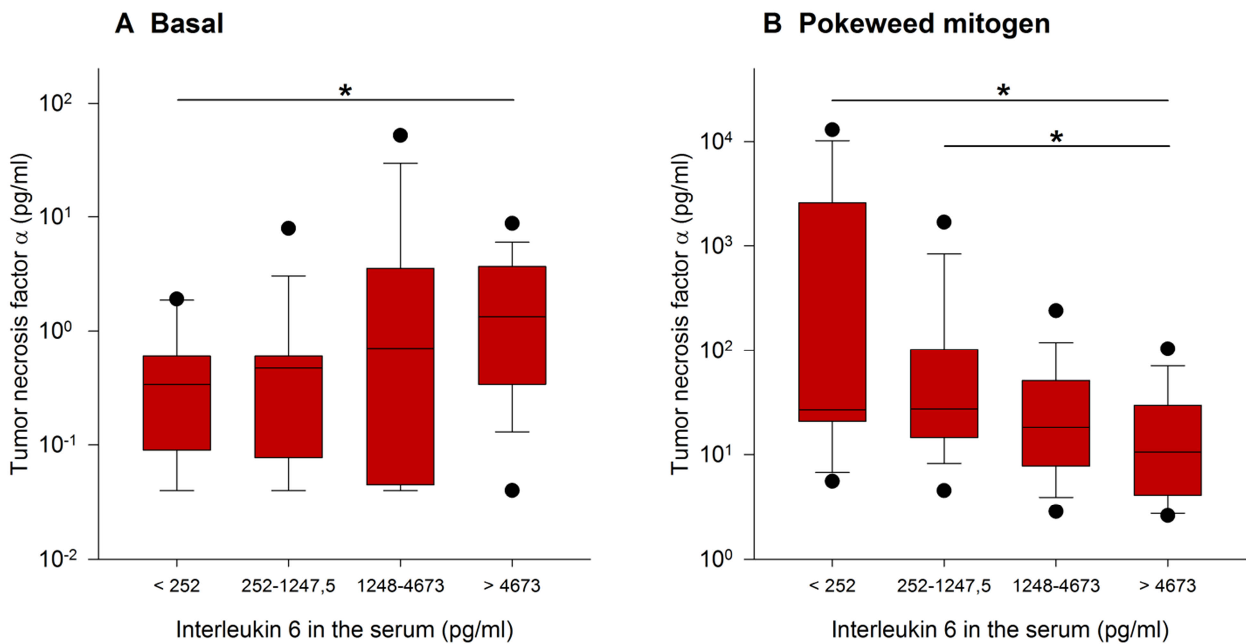
Figure 5. Relationship between serum IL-6 and IL-1β release after stimulation with LPS



The x-axis represents the serum IL-6 level, the y-axis shows the IL-1β release from whole blood after stimulation with lipopolysaccharide. A) Scatterplot with regression line (red line). Two outliers are highlighted in red. The black line represents the resulting regression line when leaving the outliers out of consideration. Note that both axes are scaled in a common logarithmic fashion. B) Patients were allocated to quartile groups depending on their IL-6 measured in the blood serum. The outliers identified in A) were included into calculation. IL: Interleukin, LPS: Lipopolysaccharide. Statistically significant differences (Mann-Whitney Rank Sum Test) are indicated as follows: * $p < 0{,}05$, *** $p < 0{,}001$.

Figure 6 shows the TNF-α release in both the unstimulated assay (*Figure 6.A*) as well as the assay stimulated with PWM (*Figure 6.B*) plotted against quartile groups for serum IL-6. While presenting the highest TNF-α concentration in the unstimulated assay, the group with the highest increase in serum IL-6 shows a considerably impaired TNF-α secretion after stimulation with PWM.

Figure 6. Relationship between serum IL-6 and TNF-α release in Basal and PWM assay



Patients were allocated to quartile groups depending on their serum IL-6 level for correlation with whole blood supernatant cytokine concentrations. A) Basal test assay (no stimulation). B) Pokeweed mitogen stimulated assay. Statistically significant differences (Mann-Whitney Rank Sum Test) are indicated as follows: * $p < 0,05$.

3.3.2.3. Disease severity classification systems

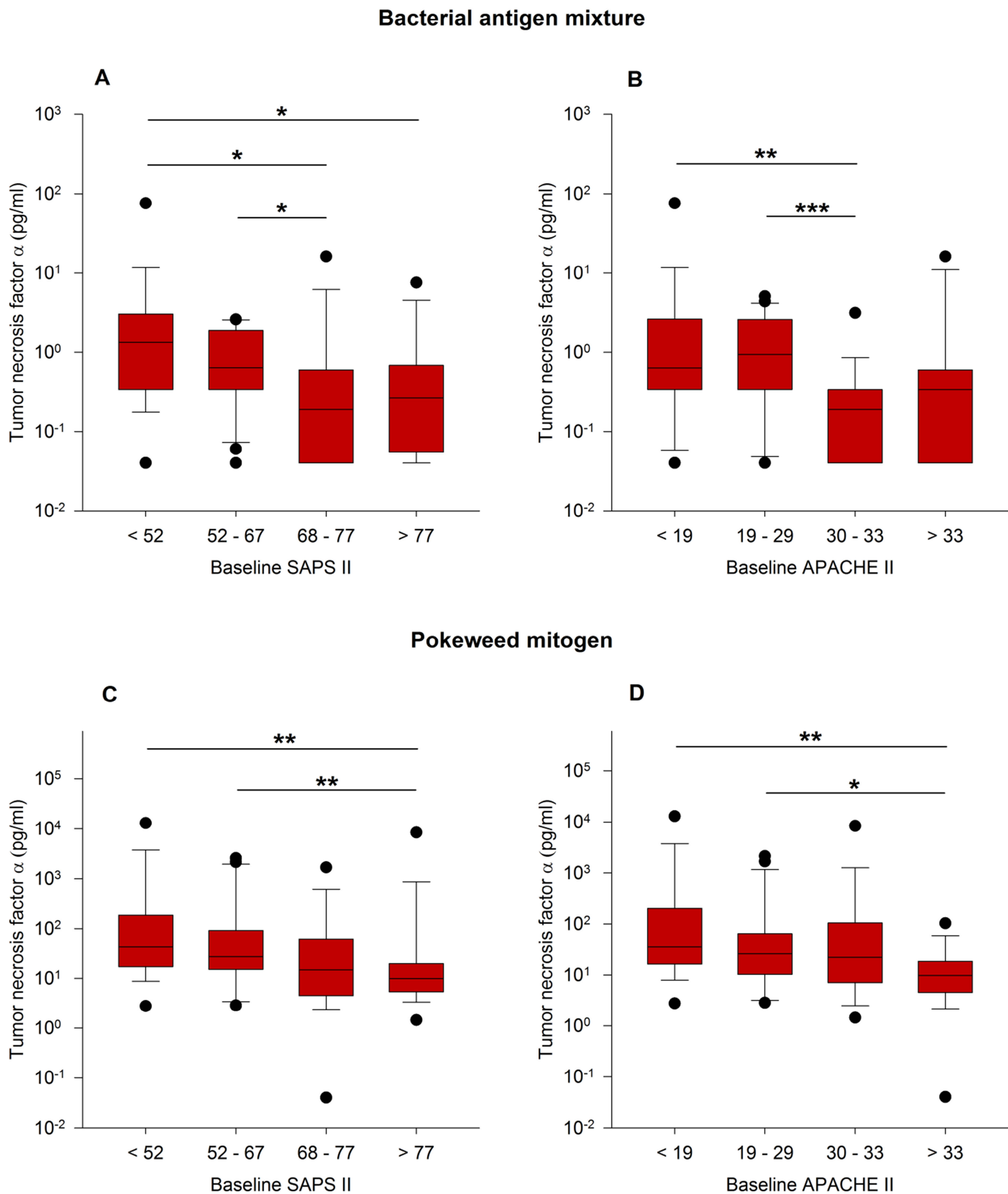
In order to determine possible correlations with disease severity, we used two disease severity classification systems which are commonly applied in critical care medicine – the Simplified Acute Physiology Score (SAPS II) and the Acute Physiology and Chronic Health Evaluation II (APACHE II). Each of these scores was calculated for the time span of 24 hours before study enrolment for any patient. If more than one value within that given time interval was documented for any variable, the value resulting in maximum score value was used for score calculation.

The SAPS II was first described in 1993 and incorporates 17 parameters, which represent physiologic measurements, information about previous health conditions and admission type: Age, heart rate, systolic blood pressure, body temperature, GCS, the presence of mechanical ventilation or CPAP, PaO_2, FiO_2, urine output, blood urea nitrogen, serum sodium, serum potassium, serum bicarbonate, serum bilirubin, WBC, the presence of chronic diseases (metastatic cancer, hematologic malignancies, AIDS) and admission type (scheduled surgical, non-scheduled surgical, medical), resulting in a score between 0 and 163. The SAPS II showed to be a good predictor of vital status at hospital discharge, independent from primary diagnosis [40]. We formed quartile groups for Baseline SAPS II score values for correlation with cytokine data.

The APACHE II score was established in 1985 and incorporates the 16 parameters age, GCS, body temperature, mean arterial pressure, heart rate, respiratory rate, FiO_2, PaO_2, arterial pH, serum sodium, serum potassium, serum creatinine, the presence of acute renal failure, hematocrit, WBC and the presence of severe organ system dysfunction or immunodeficiency, resulting in a score between 0 and 71. APACHE II proved its ability to predict mortality in a highly significant way, taking into account that, in contrast to SAPS II, the type of principal diagnosis affects mortality rates [41]. We also formed quartile groups for Baseline APACHE II score values.

In our dataset, correlation of SAPS II and APACHE II values with 90-day mortality showed a tendency towards a positive correlation, which was not statistically significant, though (data not shown). *Figure 7* shows SAPS II and APACHE II score values plotted against the release of TNF-α after stimulation with bacterial antigen mixture (*Figure 7.A, 7.B*) and PWM (*Figure 7.C, 7.D*). An increase in disease severity was associated with significantly impaired immune response both after bacterial antigen as well as after PWM stimulation. This finding applied to SAPS II as well as APACHE II scores.

Figure 7. Relationship between disease severity and TNF- α release in Bacteria and PWM assay



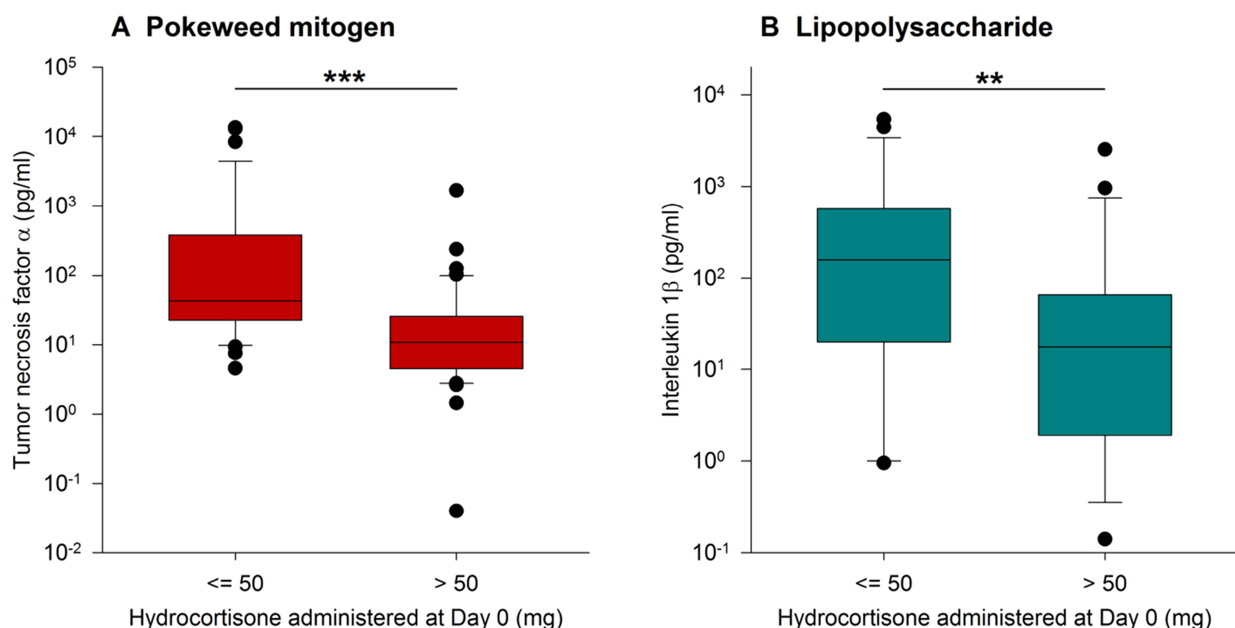
Panels A) and B) show bacterial antigen mixture assay, panels C) and D) show Pokeweed mitogen assay. TNF-α release from whole blood was correlated with quartile groups for disease severity as measured by SAPS II (A, C) and APACHE II (B, D) disease severity classification systems. TNF-α: Tumor necrosis factor α, Bacteria: Bacterial antigen mixture, PWM: Pokeweed mitogen, SAPS II: Simplified Acute Physiology Score, APACHE II: Acute Physiology and Chronic Health Evaluation II. Statistically significant differences (Mann-Whitney Rank Sum Test) are indicated as follows: * p < 0,05, ** p < 0,01, *** p < 0,001.

3.3.2.4. Hydrocortisone administration

In patients with refractory septic shock in which hemodynamic stability cannot be restored by both adequate fluid resuscitation and usage of vasopressors, it is recommended to consider the administration of corticosteroids at a dose of 200 - 300 mg of hydrocortisone per day [11, 13]. Since hydrocortisone has a dose dependent immunosuppressive effect, we excluded the patients having received minor doses of hydrocortisone. In our patient collective, 41 patients (54 %) received hydrocortisone at relevant doses of > 50 mg at Day 0. We compared this group to the patients who were administered hydrocortisone at a dose of ≤ 50 mg (most of which in fact received no hydrocortisone at all) regarding cytokine release (hydrocortisone amount: hydrocortisone > 50mg: 243,9 ± 116,7 mg/24h, n = 41; hydrocortisone ≤ 50mg: 1,8 ± 8,6 mg/24h, n = 35).

Analysis of the two hydrocortisone groups revealed no significant differences in the complete blood count, though in the disease severity scores (hydrocortisone > 50mg: SAPS II = 73,3 ± 14,8, n = 41; hydrocortisone ≤ 50 mg: SAPS II = 56,3 ± 12,8, n = 33; hydrocortisone > 50 mg: APACHE II = 31,3 ± 6,8, n = 41; hydrocortisone ≤ 50 mg: APACHE II = 22,6 ± 7,7, n = 33). Patients who received hydrocortisone at a dose of more than 50 mg had significantly higher IL-6 levels compared to the low dose group (hydrocortisone > 50 mg: serum IL-6 = 31651 ± 95995 pg/ml, n = 33; hydrocortisone ≤ 50 mg: serum IL-6 = 4581 ± 15787 pg/ml, n = 29; Mean ± SD; Mann-Whitney-U test, p = 0,016).

Figure 8. Relationship between hydrocortisone administration and cytokine release



Patients were allocated to groups of total hydrocortisone adminstered at Day 0 (<= 50 mg vs. > 50 mg) for comparison of cytokine release. A) Tumor necrosis factor α in supernatants of whole blood stimulated with Pokeweed mitogen. B) Interleukin 1β in supernatants of whole blood stimulated with lipopolysaccharide. Statistically significant differences (Mann-Whitney Rank Sum Test) are indicated as follows: ** p < 0,01, *** p < 0,001.

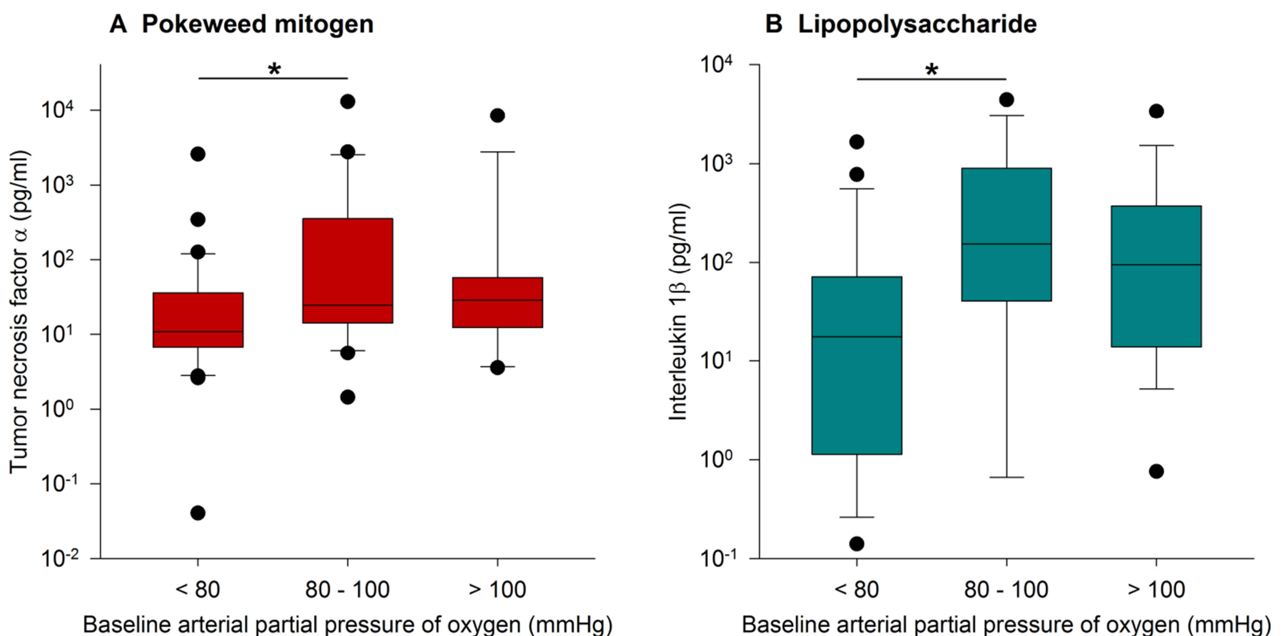
For the group of patients receiving > 50 mg of hydrocortisone, *Figure 8* shows highly significant suppression of TNFα in the PWM assay (*Figure 8*.A, $p < 0,001$) and IL-1β release in the LPS assay (*Figure 8.B*, $p < 0,01$), respectively. This significant difference was present although the stimulated cytokine response in all septic patients was enormously reduced compared to healthy volunteers.

3.3.2.5. Hypoxemia and hypoxia

Recent findings describe an anti-inflammatory effect of tissue hypoxia, which is mediated by the Adenosine A2A-receptor. This effect might be useful in preventing inflammatory tissue damage, such as in inflammatory conditions affecting the lungs or liver [45, 46], but could, on the other hand, further enhance an existing immunosuppression.

We divided the patient collective into groups of hypoxemic patients (PaO_2 < 80 mmHg), normoxemic patients (PaO_2 80 – 100 mmHg) and hyperoxemic patients (PaO_2 > 100 mmHg). SAPS II and APACHE II showed similar scores irrespective of the three PaO_2 groups (data not shown). *Figure 9* shows correlations between PaO_2 and cytokine release in the assays stimulated with PWM and LPS, respectively. In both assays, supernatant cytokine concentrations in the normoxemic group were significantly higher than those

Figure 9. Relationship between PaO2 and cytokine release



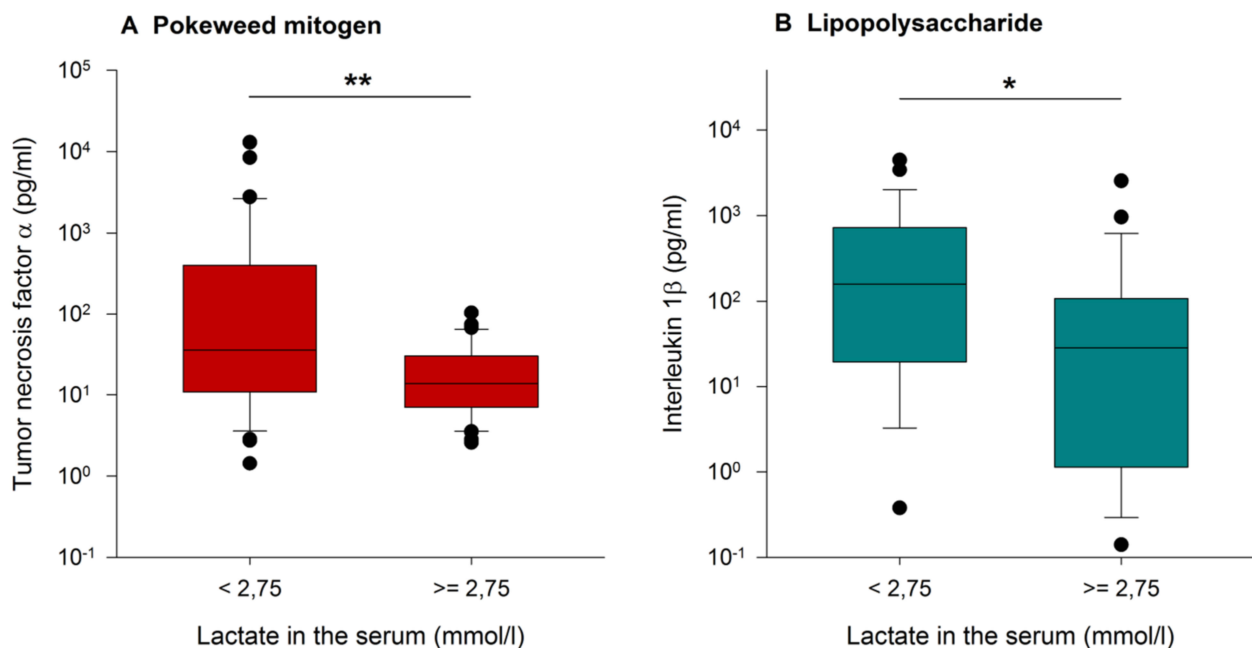
Patients were allocated to groups regarding their arterial partial pressure of oxygen (PaO_2) for comparison of cytokine release: hypoxemia (PaO_2 < 80 mmHg), normoxemia (PaO_2 80 - 100 mmHg) and hyperoxemia (PaO_2 > 100 mmHg). A) Tumor necrosis factor α measured after stimulation with Pokeweed mitogen. B) Interleukin 1β measured after stimulation with lipopolysaccharide. Statistically significant differences (Mann-Whitney Rank Sum Test) are indicated as follows: * $p < 0,05$.

in the hypoxemic group. The groups with higher oxygen tension only differed on a level that was statistically not significant.

In contrast to hypoxemia, which is defined as a lack of oxygen in the arterial blood, hypoxia is defined as an undersupply of the body tissues with oxygen. Besides PaO_2, the oxygen supply of body tissues depends on the cardiovascular system providing a certain level of tissue perfusion, which is frequently impaired in severe sepsis and septic shock. Hypoxia cannot be measured directly in the clinical routine, but since cells produce lactate when exposed to hypoxic conditions, the serum level of lactate is often used as a surrogate parameter for hypoxia.

We formed two groups, separated by the population median of maximum Baseline serum lactate level, which was 2,75 mmol/l. When investigated for Day 0 cytokine release, there showed to be significant differences in most of the test panels stimulated with PWM or LPS, with a corresponding correlation as in our analysis of hypoxemia: the group of patients supposedly suffering from hypoxia (represented by a high serum lactate level) showed an impaired release of cytokines. *Figure 10* shows the plots for TNF-α release in the PWM-stimulated assay and IL-1β release in the LPS-stimulated assay, respectively.

Figure 10. Relationship between lactate level and whole blood response



Whole blood response for the assays A) PWM TNF-α and B) LPS IL-1β for groups of patients below and above the population median for the blood serum lactate level (2,75 mmol/l). Statistically significant group differences (Mann-Whitney Rank Sum Test) are indicated as follows: * $p < 0,05$, ** $p < 0,01$.

3.3.2.6. Demographic, biometric and epidemiologic aspects

For further analysis of the patient collective regarding preexisting characteristics not directly linked to disease severity, we investigated correlations between cytokine release data and age, BMI, sex, infectious focus localization and pathogen type (gram-positive bacterial, gram-negative bacterial, viral). Concerning age and BMI, a few slightly significant differences between groups could be detected, none of which showed a consistent pattern (data not shown). None of the latter three analyses revealed a single divergence significant on the 0,05 significance level (data not shown).

3.3.3. Receiver operating characteristics

Up to this point it could be determined that firstly - in comparison to healthy individuals - our patient collective was in a state characterized by cellular immune suppression. Secondly, we could point out that the degree of immune dysfunction correlated with inflammatory parameters, established objective markers of disease severity as well as conditions exerting immunodepressive effects. Our third step was to investigate whether our method had a predictive value regarding defined clinically highly relevant endpoints.

3.3.3.1. Mortality

With seven patients who could not entirely be followed up and 14 deaths among the patients who were followed up for the whole period of 90 days, overall mortality was 20 %. By employing Receiver operating characteristic (ROC) curves, the release of IFN-γ after stimulation with PWM allowed a statistically significant prediction of overall death risk, with lower IFN-γ release indicating higher mortality (Area under the receiver operating characteristic curve (AUC) = 0,72, p = 0,01; data not shown). The other read-outs, particularly our reference read-outs PWM - TNF-α and LPS - IL-1β, did not show any significant values in the ROC-analysis regarding mortality (*Table 9, Figure 11)*.

Compared to this, the disease severity classification systems SAPS II and APACHE II also did not reveal statistically significant predictability of mortality. Altogether characterized by p-values > 0,05, AUCs were 0,61 for SAPS II Baseline and 0,64 for APACHE II Baseline (*Table 9, Figure 11*).

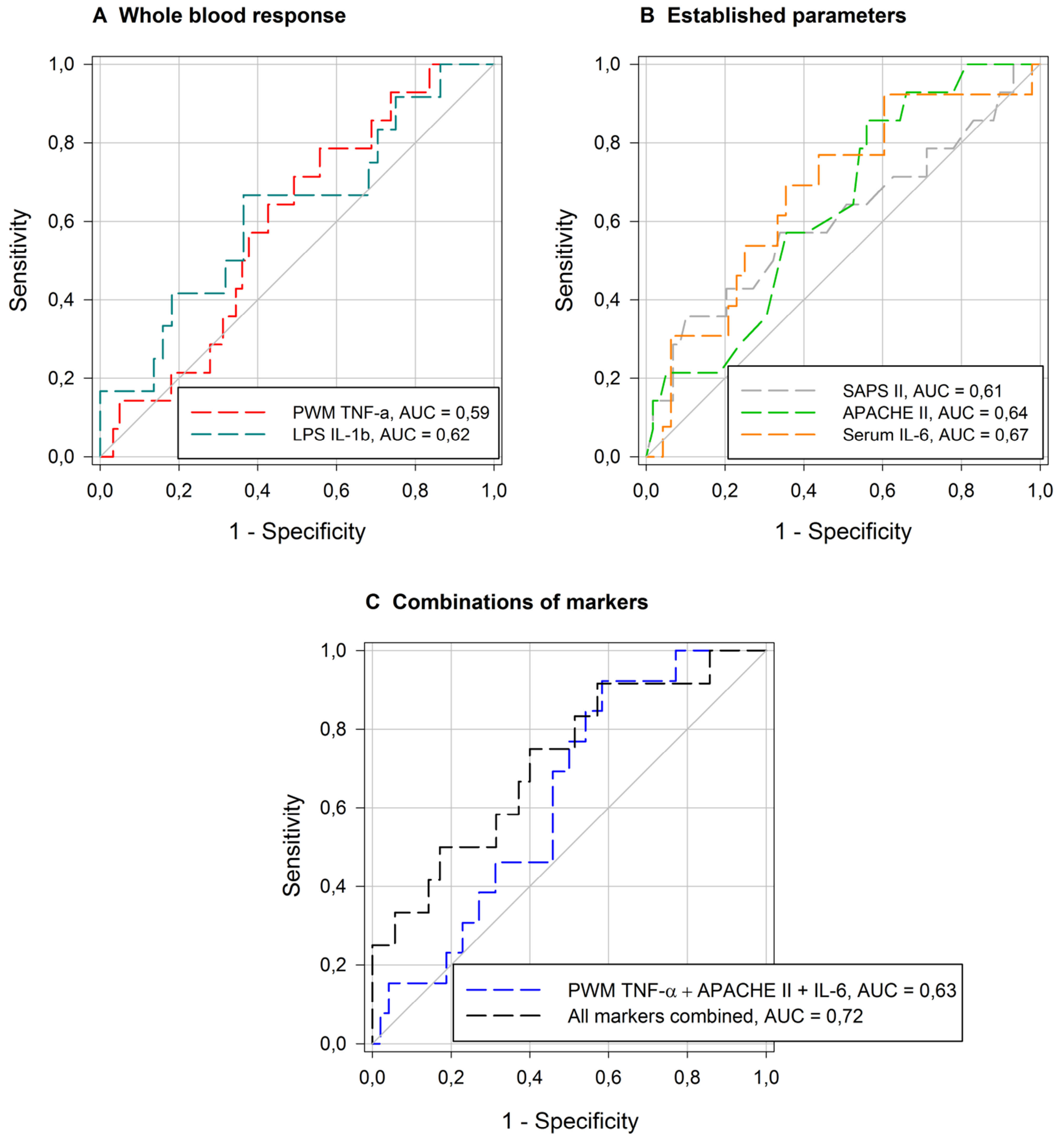
When incorporating all of the parameters (PWM – TNF-α, LPS – IL-1β, serum IL-6, APACHE II, SAPS II) by mathematically combining them in a single ROC curve, however, a result scarcely significant on the 0,05 significance level results.

Table 9. Performance of different markers in ROC-analysis regarding 90-day mortality

Marker	N	AUC (95 % CI)	p	Cut off	Sensitivity	Specificity
PWM - TNF-α	75	0,59 (0,44 – 0,75)	> 0,05	< 29,4 pg/ml	79 %	44 %
LPS - IL-1β	56	0,62 (0,43 – 0,81)	> 0,05	< 40,5 pg/ml	67 %	64 %
SAPS II	73	0,61 (0,42 – 0,79)	> 0,05	> 82	36 %	90 %
APACHE II	73	0,64 (0,49 – 0,79)	> 0,05	> 25,5	86 %	44 %
Serum IL-6	61	0,67 (0,51 – 0,84)	> 0,05	> 1195 pg/ml	77 %	56 %
PWM - TNF-α + APACHE II + serum IL-6	61	0,63 (0,47 – 0,78)	> 0,05	Fictious value	92 %	42 %
All of the above combined	47	0,72 (0,54 – 0,89)	< 0,05	Fictious value	75 %	60 %

ROC: Receiver operating characteristic, AUC: Area under the receiver operating characteristic curve, CI: Confidence interval.

Figure 11. ROC curves for the endpoint 90-day mortality



ROC-curves for A) whole blood response, B) established parameters used in sepsis and C) different combinations of markers referred to in A) and B), regarding the endpoint 90-day mortality. ROC: Receiver operating characteristic, PWM: Pokeweed mitogen, TNF: Tumor necrosis factor, AUC: Area under the receiver operating characteristic curve, LPS: Lipopolysaccharide, IL: Interleukin, SAPS II: Simplified Acute Physiology Score, APACHE II: Acute Physiology and Chronic Health Evaluation II, All markers combined: combination of PWM TNF-α, LPS IL-1β, SAPS II, APACHE II and serum IL-6.

3.3.3.2. Initiation of renal replacement therapy

Because of the rather small number of cases and the relatively low overall death rate of 20 %, we postulated another more frequent endpoint would partly compensate our lack of power and enable us to prove statistical significance. Acute renal failure (ARF) is a common complication of severe sepsis and septic shock and represents not only an indicator of disease severity, but also an independent risk factor for letality, occurring as often as 41 % in severe sepsis and septic shock in a 2007 prevalence study carried out in German ICUs [47]. According to German 2010 sepsis guidelines, indication for renal replacement therapy (RRT) in severe sepsis and septic shock has to be determined individually, but should be considered early, since diuretic agents don't improve outcome. In addition, indication for RRT in severe sepsis and septic shock should be restricted to renal failure [13], which makes the initiation of RRT a good marker for severe, RRT-dependent ARF. In our patient collective, 35 % of patients received RRT during their stay on ICU (*Table 7*).

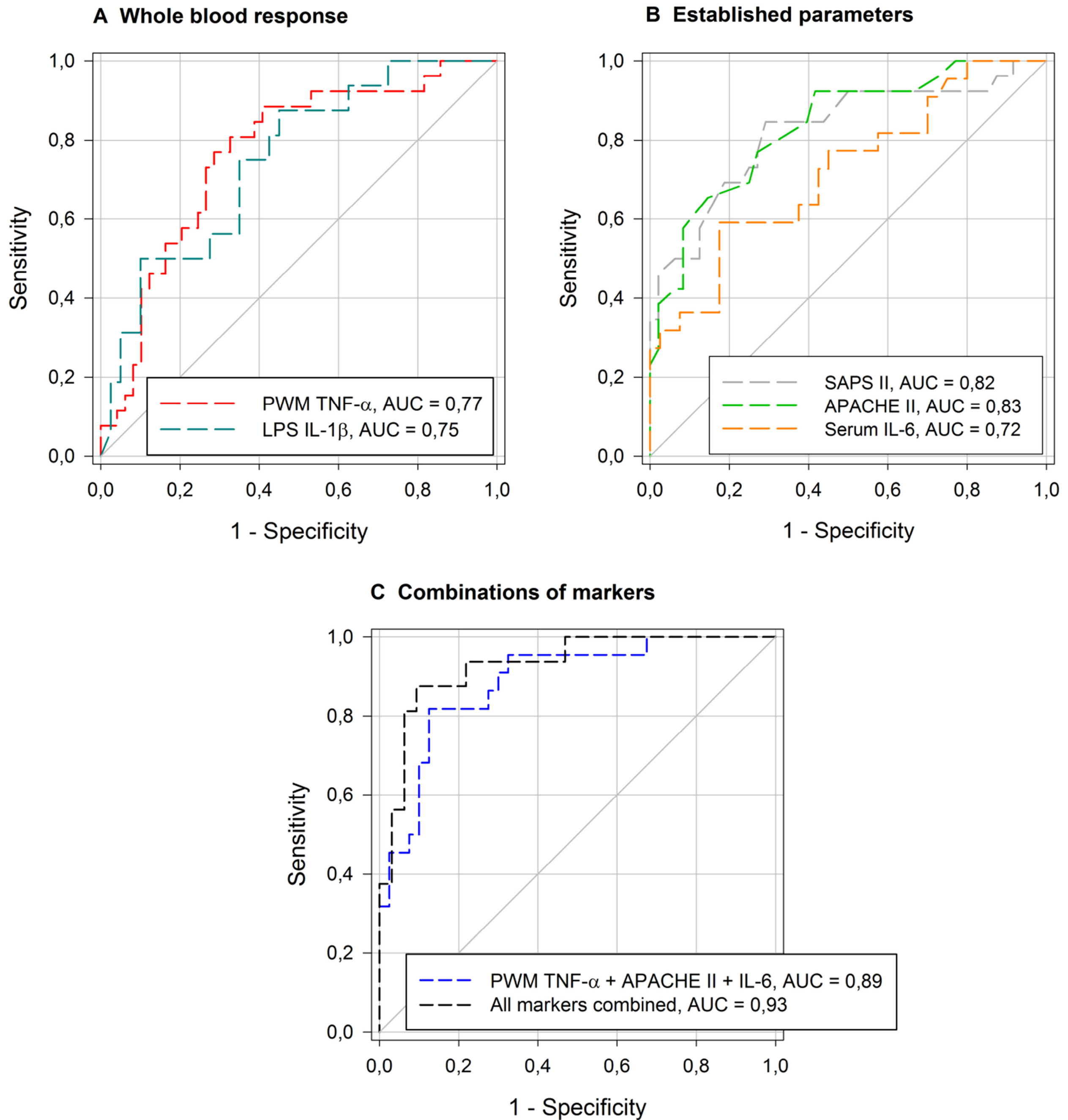
For ROC-analyses regarding the endpoint "initiation of RRT during stay on ICU", cytokine assay read-outs PWM - TNF-α and LPS - IL-1β, serum IL-6 level as well as disease severity classification systems SAPS II and APACHE II showed similar, very good results, characterized by high statistical significance (*Figure 12, Table 10*). A calculatory combination of serum IL-6 level with the cytokine read-out and disease severity classification system characterized by the best performance (namely PWM – TNF- α and APACHE II) further improved the test's performance (AUC = 0,89). When combining all of the markers (PWM – TNF-α, LPS – IL-1β, serum IL-6, APACHE II, SAPS II) gathered at the very beginning of the disease, the resulting ROC-curve reaches an AUC of 0,93 with a p < 0,0001 and a sensitivity of 88% / specificity of 91% in identifying those patients who are most likely to require RRT during their disease process (*Table 10, Figure 12*).

Table 10. Performance of different markers in ROC-analysis regarding initiation of RRT[a]

Marker	N	AUC (95 % CI)	p	Cut off	Sensitivity	Specificity
PWM - TNF-α	75	0,77 (0,65 – 0,88)	< 0,001	< 21,9 pg/ml	81 %	67 %
LPS - IL-1β	56	0,75 (0,61 – 0,89)	< 0,01	< 67,0 pg/ml	88 %	55 %
SAPS II	74	0,82 (0,72 – 0,93)	< 0,001	> 67,5	85 %	71 %
APACHE II	74	0,83 (0,74 – 0,93)	< 0,001	> 27,5	92 %	58 %
Serum IL-6	62	0,72 (0,58 – 0,85)	< 0,01	> 961 pg/ml	77 %	55 %
PWM - TNF-α + APACHE II + serum IL-6	62	0,89 (0,80 – 0,97)	< 0,0001	Fictious value	82 %	88 %
All of the above combined	48	0,93 (0,85 – 1,00)	< 0,0001	Fictious value	88 %	91 %

[a] during ICU stay; ROC: Receiver operating characteristic, RRT: Renal replacement therapy, AUC: Area under the receiver operating characteristic curve, CI: Confidence interval.

Figure 12. ROC curves for the endpoint „RRT during ICU stay“



ROC-curves for A) whole blood response, B) established parameters used in sepsis and C) different combinations of markers referred to in A) and B), regarding the endpoint „initiation of RRT during ICU stay“. ROC: Receiver operating characteristic, RRT: renal replacement therapy, PWM: Pokeweed mitogen, TNF: Tumor necrosis factor, AUC: Area under the receiver operating characteristic curve, LPS: Lipopolysaccharide, IL: Interleukin, SAPS II: Simplified Acute Physiology Score, APACHE II: Acute Physiology and Chronic Health Evaluation II, all markers combined: combination of PWM TNF-α, LPS IL-1β, SAPS II, APACHE II and serum IL-6.

4. Discussion

4.1. Evaluation of the essential findings

4.1.1. Patients versus control group

In 2011, Tamayo et al. [48] could show - in a patient collective very much resembling that of our study - that in septic shock patients plasma levels of both pro- and anti-inflammatory cytokines such as IL-6, IL-8, IFN-γ, granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-10 were significantly increased shortly after the onset of septic shock as compared to healthy controls. However, the differences in functional immune performance as assessed by a cytokine release assay in a critically ill patient collective suffering from severe sepsis or septic shock in comparison to a healthy control group revealed a severe, general cellular immune dysfunction in the patient collective. This condition was represented by - for the most part - highly significant suppression of supernatant cytokine concentrations in all of the stimulated assays except for few cytokine read-outs. In conclusion, most of the patients were already in an immunosuppressive state at the time of study enrolment, namely within 24 hours after the onset of severe sepsis or septic shock.

In SIRS, one would expect overwhelming inflammation and thus massive cytokine release exceeding the reference range defined by the healthy control group. Such a condition, however, was only present in very few patients who must be regarded as outliers considering the rest of the population. Consistent with Tamayo et al. [48], however, our patient collective simultaneously presented a severe rise in serum levels of infection parameters determined in clinical intensive care routine like CRP or IL-6. The latter is a cytokine predominantly exerting pro-inflammatory effects, such as mediating T-cell activation and an acute-phase response (and thus inducing, among others, CRP) [37]. Median Baseline IL-6 was 1248 pg/ml (*Table 8*), constituting a rise of more than 200-fold above the reference range, and none of our study patients had an IL-6 level within the reference range of < 5,9 pg/ml. Since IL-6 has a short plasma half-life, with statements in the literature ranging from minutes to hours [44, 49], it can be assumed that there was an ongoing cytokine production in vivo at the time of blood sample obtainment.

At first appearance paradoxically, however, the same individuals' in vitro whole blood cytokine response to strong immunologic stimulants was suppressed to a very low level as compared to healthy controls. Compared to the strong suppression of functional in vitro immune responses, the highly elevated plasma IL-6 levels can to some extent be explained by the fact that IL-6 is not only produced by immune cells, but also by endothelial cells, fibroblasts and keratinocytes. Therefore, in the case of such critical infectious conditions, IL-6 serves rather as a marker for tissue injury than as a specific marker for activation of the immune system [50]. Since, on the other hand, CRP is directly induced by IL-6 via the acute-phase response [51], its raised levels can also be explained by the finding above.

As a conclusion, the tremendously elevated levels of the so-called "inflammatory markers" in conjunction with the simultaneous marked cellular immune dysfunction can be characterized as an ambivalent situation. On the one hand, the whole body seems to be immunologically "on fire", with pro-inflammatory processes taking place, exponentiating themselves and thus resulting in tissue injury, organ failure and, as

a result, even more inflammation. The initial effect intentioned is not accomplished, however, since as negative feedback, anti-inflammatory processes are simultaneously initiated and upregulated, which result in severe leukocyte dysfunction. Consistently with this, recent studies tend to defeat the view of SIRS and CARS as two separate processes taking place one after another, as it was still postulated about ten years ago [52]. It is now believed that pro- and anti-inflammation are two concurrently developing processes emerging early in the pathophysiologic process [23, 48, 53], which are most commonly referred to as mixed antagonist response syndrome (MARS).

In detailed consideration of the assays and cytokines affected, our data suggest that the innate immune response (e.g. as represented by the LPS assay) seems less compromised than the specific immunity. The occurrence of marked lymphocyte apoptosis in sepsis, whose quantity is determined by disease severity [54, 55], might serve as an explanation. Consistent with this, typical changes in the differential blood count develop, resulting in relative granulocytosis and lymphopenia (median lymphocytes: 6,5 % in our patient population; reference range: 25 % - 40 %; see *Table 8*). Secondly, increased numbers of regulatory T cells (Tregs) in sepsis are to some extent responsible for decreased T cell function and proliferation [17]. Following an enhanced ingestion of apoptotic lymphocytes by macrophages, their phenotype changes to anti-inflammation, with an increase in production of IL-10 and transforming growth factor β (TGF-β) as opposed to a decrease in release of pro-inflammatory mediators like TNF-α or IL-1β [56]. Both IL-10 and TGF-β affect T cells as well as macrophages in an inhibitory way [37]. Furthermore, the complex interlinkages between the innate and acquired parts of the immune system contribute to each other's dysfunction via a lack of stimulation. Since, for example, the powerful macrophage activator IFN-γ is primarily produced by T cells, T cell apoptosis or dysfunction will secondarily result in macrophage dysfunction [57]. As a conclusion, initial lymphocyte apoptosis occurring early in sepsis results in the initiation of anti-inflammatory processes and discontinuation of immunostimulating processes, and thus secondarily also inhibits the innate immune system.

An emphasis on anti-inflammatory processes can be reconstructed in our data using IL-10 release values. In the patient population, IL-10 secretion was less impaired than the release of pro-inflammatory cytokines (*Figure 3*). It is notable, however, that in all of our assays, the IL-10 release in severe sepsis or septic shock was either similar or even slightly compromised compared to the control group. An IL-10 secretion significantly exceeding the control group could not be demonstrated in any of our cytokine release assays. Still, as a result from massive impairment of pro-inflammation opposed to mild impairment of anti-inflammation, the state of equilibrium shifts to anti-inflammation, which might be contributing to the state of immunosuppression in sepsis.

For the Bacteria and Fungi assays, the high variability between patients regarding their whole blood response could be attributed to differences in prior antigen exposure, since the intensity of antigen exposure determines the quality and quantity of a subsequent immune response to the same antigens. A similar variability - although not quite as highly developed - is also present in the assays using unspecific stimulants, which do not depend on prior antigen exposure (*Figure 2, Figure 3*). Other studies have confirmed the presence of a high interindividual variability in cytokine release from whole blood [58]. However, each person seems to have a defined individual whole blood response, which is - within rather narrow limits - constant over the course of several weeks. It is postulated that the individual patient's HLA-

DR genotype, which is known to correlate with cytokine release, might play an important role [58]. This finding limits the interindividual comparability of whole blood response, imposes caution in interpreting the data and in fact complicates proving significant differences between independent samples. Instead, it suggests the monitoring of whole blood release kinetics for paired groups as a promising concept for further studies.

4.1.2. Subcohort-analyses

As pointed out in **3.2.2**, in assays addressing solely acquired immune pathways, no further sub-cohort analysis was possible due to a general suppression below the lower detection limit in the majority of cytokines and for most stimulation assays. Discrimination was possible, though, only in assays using strong polyclonal stimulants, such as LPS and PWM. In order to maintain comparability between single analyses - and to restrict the extent of this work to a usual dimension - we mostly limited the shown data in this field to two key cytokine read-outs, IL-1β and TNF-α, as measured from the assays stimulated with the substances named above – LPS and PWM. Both cytokines, IL-1β as well as TNF-α, are predominantly released by macrophages and mediate pro-inflammatory effects such as inducing fever, stimulating nitric oxide and prostaglandin synthesis and activating other immune cells. Furthermore, both cytokines induce each other's and IL-6 synthesis and potentiate each other's actions. They have very similar effects and are responsible for many of the constitutional responses to acute inflammation in innate immunity [15].

4.1.2.1. Inflammatory markers

Patients with moderate leukocytosis showed the best whole blood immune response, whereas both leukopenia as well as severe leukocytosis were associated with increased immune dysfunction (*Figure 4*). One possible explanation for this is the consideration of WBC as a marker of disease severity. Patients at both extreme ends of the spectrum - in leukopenia as well as severe leukocytosis - presumably are affected more seriously than patients with normal WBC or a moderate degree of leukocytosis, which serves as a physiologic reaction to an infection. Our data suggest that those patients with a higher disease severity show the worst whole blood immune response, which is consistent with our other findings.

Another approach might be the duration of the infection, which was shown to be of high interest for the prediction of disease progression in a microsimulation model [59]. Owing to our study design, there is considerable difficulty in interpreting the time axis regarding the onset of (non-severe) sepsis, bacteremia or infection. None of the onsets of either of these conditions was documented in our study protocol, nor is in general easy to determine in clinical routine. The only condition documented was the fulfillment of severe sepsis or septic shock definition criteria, within 24 hours after which study enrolment was conducted. In the literature, there is relatively little information about the exact kinetics of septic conditions and inflammatory response, but murine sepsis models based on cecal ligation and puncture (CLP) showed that the systemic inflammatory response represented by elevated levels of pro-inflammatory cytokines in the blood plasma began to emerge within 2 – 8 hours after the insult, depending on the individual cytokine [60]. Anti-inflammatory processes are assumed to begin within the first 24 hours in human sepsis [61]. A possible hypothesis would be that those patients presenting a moderate rise in infection parameters and a better whole blood response were still in an earlier state shortly after onset of the infection, in which anti-inflammation and corresponding immune suppression was not as bold as in those patients in more progressed states.

As already pointed out (in **4.1.1**), the ambivalent behavior of IL-6 serum levels and corresponding whole blood response as demonstrated in *Figures 5 and 6* can be attributed to the finding that in progressed phases of infectious conditions, the main incentive for the resulting "cytokine storm" is not a physiologic

reaction to pathogens but the massive liberation of so-called damage-associated molecular patterns (DAMPs). These are released following cellular injury, mainly from the human mitochondria, and have a high genetic and thus structural resemblance to the bacterial surface. The latter acts in a very similar way and constitutes the physiological activator of the immune response; it goes by the name pathogen-associated molecular pattern (PAMP) [62-64].

In summary, it can be stated that the degree of tissue injury and thus the disease severity, represented, among others, by a marked rise in inflammatory markers such as CRP and IL-6, is negatively correlated with the whole blood response to immunologic stimulants. This results in a heightened susceptibility to secondary infection and thus late-phase mortality.

4.1.2.2. Disease severity classification systems

Consistent with the findings named above, patients in higher disease severity - as quantified by the common disease severity classification systems SAPS II and APACHE II – were shown to have a significantly impaired whole blood response as compared to patients with less marked disease severity (*Figure 7*). This finding applied to the PWM - TNF-α assay as well as for the bacterial antigen mixture - TNF-α assay, which was included in *Figure 7* for this reason. The LPS - IL-1β assay showed an analogous trend, but did not reach statistical significance (data not shown).

Both of the applied scores are constructed in such a way that they mainly incorporate physiologic parameters which are typically deranged in case of organ failure and tissue injury – such as serum electrolytes and neurologic, hemodynamic, respiratory, renal and hepatic organ failure parameters [40, 41]. As a conclusion, the results included in this section are equivalent to the aforementioned consideration of inflammatory parameters, using another method of quantifying the abstract term "disease severity".

4.1.2.3. Hydrocortisone administration

Figure 8 reveals the highly significant suppression of pro-inflammatory cytokine release in the group of patients receiving relevant doses of hydrocortisone. Considering the indications as well as the effects of hydrocortisone, an ambivalent situation emerges.

First of all, the pharmacologic effects exerted by hydrocortisone must be taken into account. Hydrocortisone has immunosuppressive effects mediated in a number of different ways, and thus causes considerable impairment in whole blood response. Since it's pharmacokinetic elimination is dependent on renal and hepatic function [65], it can be assumed that there is pharmacologic activity for the whole in vitro incubation period of 48 hours.

But one must also take into account that according to guidelines, the application of hydrocortisone should

be restricted to patients in refractory septic shock, in which hemodynamic stability cannot be restored by both adequate fluid resuscitation and usage of vasopressors [11, 13]. Within our patient collective, this condition affects those patients in the most progressed phase of septic shock and defines a high severity of disease. Accordingly, the group of patients receiving > 50 mg of hydrocortisone showed to have considerably higher SAPS II and APACHE II scores than the group of patients who did not receive relevant doses of hydrocortisone. In part, the differences between the two groups could thus be caused by patient selection in accordance with the indication for hydrocortisone therapy in septic shock.

4.1.2.4. Hypoxemia and hypoxia

As demonstrated in *Figure 9*, hypoxemia significantly suppressed the immune response in our study population. The same goes for elevated levels of serum lactate as a surrogate parameter for hypoxia, accordingly. Choukèr et al. could show that there is a hypoxia-induced anti-inflammatory mechanism mediated by the A2 adenosine receptor (A2AR), which results in a reduction of lung tissue damage in patients with acute respiratory distress syndrome (ARDS) as well as the systemic effect of reducing inflammatory liver injury in A2AR-competent mice [45, 46]. These anti-inflammatory effects are accomplished by different mechanisms including the inhibition of oxidative burst, a reduction in platelet activation and thus in microvascular occlusion, a reduced release of pro-inflammatory cytokines such as TNF-α or IFN-γ opposed to an increased release of anti-inflammatory cytokines such as IL-10 or IL-4 and thus a shift of the lymphocyte TH_1/TH_2-equilibrium towards the TH_2-pathway [66, 67]. Adequate oxygenation thus seems like an important component of a profound sepsis therapy.

Caution is imposed, however, regarding the causality of effects. Especially considering the serum lactate level, not only respiratory insufficiency can be made accountable for its rise. Circulatory failure resulting in tissue hypoperfusion as well as tissue damage also result in increased lactate levels, two conditions which typically characterize progressed septic conditions independently from the respiratory situation.

4.1.3. Receiver operating characteristics

Our reference cytokine read-outs PWM – TNF-α and LPS – IL-1β provided results comparable to multifactorial disease severity classification systems and an established inflammatory marker determined in blood serum (IL-6) in predicting different outcome measures (as shown in *Tables 9 - 10* and *Figures 11 - 12*).

In the case of 90-day mortality, however, no isolated marker was capable of providing statistically significant results. This is most likely due to our small sample size combined with the fact that our patient population presented a low overall mortality of 20%, one third of which represented late phase mortality characterized by death causes associated with complications in the disease process and in some cases even comorbidities with no or little connection to sepsis. Still, the mathematical combination of all markers used provided a statistically significant result allowing a prognosis for the chances of death at the very beginning of the disease.

These results could be considerably improved when looking at the presence of marked acute renal failure in the course of the disease, represented by the initiation of renal replacement therapy. This is a common complication of severe septic conditions and thus more closely associated with the severity of disease progress than overall mortality, which tends to be confounded by various independent variables. In consideration of the endpoint "initiation of renal replacement therapy in the disease progress", all of the parameters showed a good predictive value with high statistical significance. These solitary results could yet be outperformed by a calculatory combination of different markers, resulting in p-values < 0,0001 and an area under the receiver operating characteristic curve of up to 0,93.

These findings suggest that the whole blood response to strong immunologic stimuli might be a powerful supplement to established markers and scoring systems in sepsis. The combination of parameters showed to have eminent prognostic value in our study population. Solitary parameters, even though characterized by a strong positive correlation among one another, seem to complement each other. In clinical routine, this could be used to identify patients at high risk for an especially severe clinical course and to adjust different treatment strategies early, as specified in the following section.

4.2. Conclusions for diagnostics and therapy in sepsis

Besides the standard therapy for sepsis - including early anti-infective therapy, focus control and adjunctive therapies aiming at stabilizing the patient's vital functions - a variety of different therapeutic approaches targeting the immune response have been tested. The focus of these interventions originally lay on counteracting the hyperinflammatory cytokine storm by immunomodulation in a negative way, i.e. by administering antibodies directed against TNF-α or other pro-inflammatory cytokines [68], absorption of cytokines from the blood plasma [69] or hydrocortisone administration in high as well as in low doses [17]. Up to the present day, none of these attempts showed to be promisingly successful regarding mortality in controlled clinical trials. With the focus of clinical research concentrating on the immunosuppressive state characterizing the course of sepsis, recent trials included the contrary approach of immunostimulation, using for example granulocyte-macrophage colony-stimulating factor (GM-CSF) or IFN-γ in patients with verified immune dysfunction [70-72]. Despite being a concept with first promising results, dating as far back as to the year 1997 [71], conclusive results supporting this approach are still pending to the present day due to a lack of sufficiently powered controlled clinical trials.

4.2.1. Hygiene and reverse isolation

Our data suggest that from the first moments after onset of septic shock, immune competence is severely limited. The degree of this immunologic dysfunction positively correlates with disease severity, levels of inflammatory parameters, presence of hypoxemia and administration of hydrocortisone. Thanks to modern intensive care medicine, most patients treated in an ICU nowadays survive the first phase of septic shock characterized by hypotension and sequential organ failure due to supportive measures compensating circulatory and organ failure. A notable number of deaths, however, occur in later phases of the disease course and can be attributed to the sequelae of immunosuppression [57]. It could be demonstrated that patients suffering from critical illness and associated immune suppression are target to secondary infections not only by virulent organisms like Clostridium difficile or Staphylococcus aureus but also by opportunistic pathogens like Stenotrophomonas maltophila, Acinetobacter calcoaceticusbaumannii and Candida albicans as well as reactivation of cytomegalovirus (CMV) and herpes simplex virus (HSV) [57, 73-75].

An expansion of antiinfective treatment does not seem appropriate when facing this issue. As soon as one organism is defeated by antibiotics, antimycotics or virostatic agents, another one which is not affected by the drugs applied will lead to a superinfection [57], which is especially favored by the fact that those pathogens tend to be multidrug-resistant [75]. In order to minimize the hazard of acquiring an opportunistic infection, first of all more basic methods seem promising. Patients for sure do benefit from strict compliance to hygienic regulations. More efforts should be taken to create a better awareness for this issue in the field of medical staff, especially in ICUs. Moreover, in the patients most at risk for secondary infection, temporary reverse isolation - as is typically employed in other patient collectives in immunosuppression - might have beneficial effects, though a recent review was able to show that the

incidence of errors in drug administration, a delay in examinations such as CT-scans, noncompliance and adverse events was increased in patients under isolation [76].

4.2.2. Restriction of hydrocortisone administration

Secondly, the indication for applying hydrocortisone in sepsis should be strictly questioned, since it can be considered capable of further weakening the immune response. Both high-dose as well as low-dose administration of corticosteroids were performed in previous times, none of which showed conclusive improvement in survival [77, 78]. There exists some evidence, however, that an earlier reversal of shock may be accomplished by corticosteroid application [78-80]. A 2008 randomized controlled trial showed that neither a survival benefit nor an increase in the rate of shock reversal could be accomplished by administration of hydrocortisone in septic shock. It could yet be demonstrated that in those patients experiencing shock reversal, shock reversed earlier under treatment with hydrocortisone, entailing higher rates of secondary infections including a recurrence of septic shock, however [80].

Hence, in the latest guidelines, only minor recommendation exists for corticosteroid administration, which is restricted to cases of refractory septic shock which do not respond adequately to fluid resuscitation and vasopressor therapy [11, 13]. In summary it can be said, therefore, that if possible, the use of corticosteroids in sepsis should be re-visited on its benefits and risks to potentially prevent possible detrimental effects to the immune system.

4.2.3. Immune function monitoring and modulation

Another approach strongly suggested by our study data, which is being increasingly discussed lately, implies actually reactivating the depressed immune system, guided on the basis of patients' actual immune status. Previous approaches to immune monitoring in sepsis implied the measurement of HLA-DR expression on monocytes and, similar to the method applied in our study, of in vitro TNF-α release in whole blood following stimulation with LPS. In patient collectives initially being in an immunosuppressed state as measured by the criteria named above, the application of GM-CSF or IFN-γ, respectively, was able to restore monocytic HLA-DR expression as well as the in vitro whole blood response besides showing clinical benefits as far as can be assessed from the published data [71, 72]. These early studies were neither randomized trials nor sufficiently powered to demonstrate survival benefits, though. In a meta-analysis from 2011, no significant effect from the administration of granulocyte colony stimulating factor (G-CSF) or GM-CSF on 28-day mortality could be shown. In the therapy groups, however, a significant increase in the rate of reversal from infection could be demonstrated [81]. However, a critical review addressing the meta-analysis mentioned above faults methodical deficiencies and calls for subsequent sufficiently powered randomized controlled trials to prove a mortality reduction [82]. The latest review on the topic, published in 12/2015, came to the conclusion that in the absence of deleterious side

effects by GM-CSF administration in sepsis patients, multiple clinical benefits such as more rapid recovery from infection, a decrease in length of hospital stay and in requirement of mechanical ventilation could be shown [83]. A conclusive prove of mortality reduction could not be delivered up to the present day, however, which might be attributed to inconsistencies in patient screening for signs of immune suppression, missing differentiation of patient populations and the absence of long-term follow-up [83]. Nevertheless, immune stimulatory therapies in sepsis are a promising new concept and seem eligible to become the next milestone in sepsis therapy advancements.

Another implication from our study data is the implementation of new risk stratification systems utilizing cytokine release data in order to assess prognosis regarding the disease progress. As we could show in the ROC-section, whole blood response data were equivalent in performance to established sepsis markers and disease severity classification systems and their addition eminently improved performance of ROC analyses.

4.3. Limitations and considerations

4.3.1. No adjustment for confounding variables

First of all it is notable that we did not adjust for confounding variables. Having a closer look at the results section for inter-patient correlations (3.2.2), it is apparent that most of the parameters examined show a correlation with disease severity, which could be regarded as a confounding variable in this context. More severely affected individuals tend to show more distinct alterations when it comes to inflammatory parameters, are more likely to fulfill the criteria for an indication for hydrocortisone, tend to be more impaired in regard to respiration and gas exchange with consecutive hypoxemia and hypoxia and obviously show higher scores in the disease severity classification systems applied. In conclusion, it is not legitimate to attribute between-group differences in immune response solely to the one parameter investigated in the individual case, and such comparisons have to be treated with caution.

4.3.2. Cytokine values partly below the lower detection limit

Secondly, as mentioned in the methods section, in some assays most of the cytokine release values in the patient population showed values out of range below, i.e. below the lower detection limit. In order not to eliminate most of the values for the concerned assays, we used the lower detection limit in place of individual values, which were not providable with the laboratory methods applied. This missing differentiation in the low value range, affecting a substantial portion of the values incorporated in our calculations, to some extent limits the statistical power as well as the validity and interpretation of the data in this very low range.

4.3.3. Missing information about the time of infection onset

Owing to our study design, the only specific moment in time documented was the onset of severe sepsis or septic shock, marked by beginning organ failure or hypotension, 24 hours after which study enrolment had to be conducted. The period of time in which infection was already present before fulfilling the severe sepsis/septic shock criteria, however, cannot be reconstructed from our data. This limits comparability between single patients and especially statements concerning the time scale of immunologic processes in sepsis.

4.3.4 Consideration of the new Sepsis-3 definitions

With the SISPCT trial being conducted before publication of the Sepsis-3 definitions [4, 25], the inclusion criteria and thus data analysis naturally were based on the former ACCP/SCCM definitions [2] valid at that time. We thus did not considerate Sepsis-3 definitions in the course of this work. It is to say, however, that our findings on the behavior of immune function in sepsis and the importance of end organ failure for the progression of sepsis are supported by the statements of the Sepsis-3 publication [4].

5. Summary

Despite great efforts on research on sepsis, it is still among the leading causes of death [1] and constitutes the leading cause of morbidity and mortality of patients dependent on intensive care treatment [84]. Most deaths due to sepsis occur in later phases, days after the onset of the disease [85], and seem to be attributable to sepsis-induced alterations of the immune response [84, 86]. In order to closer investigate the alterations affecting the immune system in severe septic conditions, we prospectively enrolled 76 patients with severe sepsis or septic shock, respectively, and eleven healthy individuals as a control group. For the purpose of monitoring their cellular leukocyte responsiveness, whole blood withdrawn shortly after the onset of severe sepsis or septic shock, respectively, was incubated for 48 hours without stimulation or stimulated with different agents addressing the innate or acquired parts of the immune system. Subsequently, supernatant cytokine concentrations were measured. Results were correlated with clinical data.

As opposed to the control group, the patient population presented marked immune dysfunction with an emphasis on the acquired immune system. In contrast, anti-inflammatory markers were relatively upregulated. Within the patient population, those with higher inflammatory markers, higher disease severity scores, exposure to hypoxemia/hypoxia and hydrocortisone administration showed a more pronounced decrease in immune function. Whole blood response data showed similar performance to established markers and classification systems in predicting those patients with fatal outcome and those in future need of renal replacement therapy due to acute renal failure. The different markers seem to complement each other and in combination provided a superior performance.

The view of systemic inflammation and anti-inflammation as two separate processes developing subsequently has to be reconsidered in favor of being two concurrently developing processes emerging early within the disease progress. Further advances in sepsis therapy with focus on immune status are needed to improve outcome. Those should incorporate adequate oxygenation, hygienic measures and consideration of reverse isolation, strict indication for corticosteroids and especially immunomodulatory therapies restoring immune function.

6. Zusammenfassung in deutscher Sprache

Trotz großer Forschungsaufwendungen rangieren septische Erkrankungen nach wie vor weit oben in den Todesursachenstatistiken [1] und stellen die häufigste Ursache für Morbidität und Mortalität für auf Intensivstationen behandelte Patienten dar [84]. Die meisten Sepsis-bedingten Todesfälle sind in der Spätphase, Tage nach dem Ausbruch der Erkrankung, zu verzeichnen [85], und scheinen in Zusammenhang mit durch die Erkrankung bedingten Veränderungen der Immunantwort zu stehen [84, 86]. Um diese im Rahmen schwerer septischer Erkrankungen auftretenden Veränderungen näher zu untersuchen wurden prospektiv 76 Patienten mit schwerer Sepsis bzw. septischem Schock und elf gesunde Kontrollen rekrutiert. Zum Zweck der Untersuchung ihrer zellulären Immunität wurde kurz nach Einsetzen der schweren Sepsis bzw. des septischen Schocks Vollblut gewonnen und für 48 Stunden nativ oder nach Versetzen mit verschiedenen Stimulanzien der angeborenen sowie der erworbenen Immunität inkubiert. Anschließend wurden die Zytokinkonzentrationen im Überstand gemessen und die Ergebnisse mit klinischen Patientendaten in Beziehung gesetzt.

Verglichen mit der Kontrollgruppe zeigte das Patientenkollektiv eine ausgeprägte Dysfunktion der Immunantwort mit Betonung des erworbenen Immunsystems. Gegensätzlich hierzu stellte sich eine relative Hochregulation anti-inflammatorischer Parameter dar. Innerhalb des Patientenkollektivs war eine ausgeprägtere Einschränkung der Immunantwort bei jenen Patienten nachweisbar, die höhere laborchemische Entzündungsparameter und eine höhere Krankheitsschwere aufwiesen sowie Hypoxämie bzw. Hypoxie ausgesetzt waren und therapeutisch Hydrokortison verabreicht bekamen. Die von uns erhobenen Daten zur zellulären Immunität zeigten sich in der Vorhersage letaler Verläufe sowie der Notwendigkeit von Nierenersatztherapie aufgrund akuten Nierenversagens im weiteren Krankheitsverlauf etablierten Scoring-Systemen zur Krankheitsschwereeinschätzung sowie etablierten Sepsis-Markern nicht unterlegen bzw. scheinen sich gegenseitig zu ergänzen. In Kombination zeigten die verschiedenen Marker ein jedem einzelnen Marker deutlich überlegenes Ergebnis.

Die Betrachtung von Inflammation und Anti-Inflammation als zwei getrennte, nacheinander ablaufende Prozesse muss zugunsten eines Modells der Koexistenz zweier sich parallel bereits im frühen Krankheitsverlauf entwickelnder Prozesse überdacht werden. Weitere Fortschritte in der Therapie der Sepsis mit dem Hauptaugenmerk auf Immunprozessen sind notwendig, um die Prognose weiter zu verbessern. Diese sollten eine ausreichende Oxygenierung, die Optimierung der Krankenhaushygiene sowie die Erwägung von Umkehrisolationsmaßnahmen, eine strenge Indikationsstellung für Kortikosteroide und insbesondere immunomodulatorische Therapieansätze, die auf die Wiederherstellung der Immunkompetenz ausgerichtet sind, beinhalten.

7. List of abbreviations

°C	Degrees Celsius
µg	microgram
µl	microliter
A2AR	A2A adenosine receptor
ACCP	American College of Chest Physicians
ALAT	Alanine aminotransferase
ANOVA	Analysis of variance
APACHE II	Acute Physiology And Chronic Health Evaluation II
ARDS	Acute respiratory distress syndrome
ARF	Acute renal failure
ASAT	Aspartat aminotransferase
AUC	Area under the (receiver operating characteristic) curve
Bacteria	Bacterial antigen mixture
BE	Base excess
BMI	Body Mass Index
bpm	Beats per minute
CARS	Compensatory anti-inflammatory response syndrome
CBA	Cytometric bead array
CD	Cluster of Differentiation
CD3/28	Human T-activator CD3/CD28

CI	Confidence interval
CLP	Cecal ligation and puncture
CMV	Cytomegalovirus
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
FiO_2	Fraction of inspired oxygen
Fungi	Fungal antigen mixture
GCS	Glasgow Coma Scale/Score
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOT	Glutamic oxaloacetic transaminase
GPT	Glutamate-pyruvate transaminase
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
HV	Healthy volunteer
ICU	Intensive care unit
IFN	Interferon
IL	Interleukin
IQR	Interquartile range
JAMA	Journal of the American Medical Association
kPa	kilopascal
LPS	Lipopolysaccharide
MAP	Mean arterial (blood) pressure

MARS	Mixed antagonist response syndrome
MFI	Mean fluorescence intensity
ml	millilitre
ml/kg/h	Millilitres per kilogram body weight per hour
mm^3	Cubic millimeters
mmHg	Millimeters of mercury
mmol/l	millimoles per liter
MODS	Multiple organ dysfunction syndrome
n	Number of cases
NASA	National Aeronautics and Space Administration
$paCO_2$	Arterial partial pressure of carbon dioxide
PAMP	Pathogen-associated molecular pattern
PaO_2	Arterial partial pressure of oxygen
PCT	Procalcitonin
PMA	Phorbol 12-myristate 13-acetate
ROC	Receiver Operator Characteristics
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RRT	Renal replacement therapy
SAPS II	Simplified Acute Physiology Score II
SCCM	Society of Critical Care Medicine
SD	Standard deviation

Sepsis-3	The third international consensus definitions for sepsis and septic shock
SIRS	Systemic inflammatory response syndrome
SISPCT	Placebo Controlled Trial of Sodium Selenite and Procalcitonin Guided Antimicrobial Therapy in Severe Sepsis
SS	Study patients suffering from severe sepsis or septic shock
Th	Helper T cell
TLR	Toll like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
US, USA	United States (of America)
WBC	White blood count

8. Register of figures

Figure 1: Dependency of survival on the early initiation of antimicrobial therapy. From: Kumar et al., 2006 [12]

Figure 2: Patients versus control group in unstimulated assay and after stimulation with recall antigens and PWM

Figure 3: Patients versus control group after stimulation with LPS, PMA and CD3/28

Figure 4: Relationship between white blood count and IL-1β release after stimulation with LPS

Figure 5: Relationship between serum IL-6 and IL-1β release after stimulation with LPS

Figure 6: Relationship between serum IL-6 and TNF-α release in Basal and PWM assay

Figure 7: Relationship between disease severity and TNF-α release in Bacteria and PWM assay

Figure 8: Relationship between hydrocortisone administration and cytokine release

Figure 9: Relationship between PaO2 and cytokine release

Figure 10: Relationship between lactate level and whole blood response

Figure 11: ROC curves for the endpoint 90-day mortality

Figure 12: ROC curves for the endpoint “RRT during ICU stay”

9. Register of tables

Table 1: Definition criteria for sepsis-associated conditions

Table 2: SISPCT criteria for severe sepsis and septic shock

Table 3: Demographic and biometric characteristics of patients versus control group

Table 4: Sepsis and admission type characteristics

Table 5: Microbiological and infection characteristics

Table 6: Baseline medical status

Table 7: Outcome characteristics

Table 8: Baseline laboratory values

Table 9: Performance of different markers in ROC-analysis regarding 90-day mortality

Table 10: Performance of different markers in ROC-analysis regarding initiation of RRT

10. Partial aspects published

1. Kaufmann, I., Sudhoff, L., Feyahn, M., Goeschl, J., Hoppstock, C., Koehler, M., Sams, C., Feuerecker, M., Briegel, J., Chouker, A., *A new ex vivo cytokine release assay as a monitoring tool for immune answer in patients with septic shock.* American Society of Anesthesiologists 2013 Annual Meeting, San Francisco, California, USA, 10/2013

2. Sudhoff, L., Kaufmann, I., Feuerecker, M., Crucian, B., Sams, C., Mehta, S., Pierson, D., Schelling, G., Choukèr, A., *From space to the septic patient: assessment of cellular immunity in severely immune compromised conditions.* NASA Human Research Program Investigators' Workshop, Galveston, Texas, USA, 02/2016

3. Sudhoff, L., Feuerecker, M., Kaufmann, I., Crucian, B., Sams, C., Mehta, S., Pierson, D., Schelling, G., Choukèr, A., *Cellular immunity in immune compromised conditions – from space to the intensive care unit (Hospace-Study)*, Young researcher session, joint life science meeting: life in space for life on earth, European Space Agency, Toulouse, France, 06/2016

4. Bloos, F., Trips, E., Nierhaus, A., Briegel, J., Heyland, D.K., Jaschinski, U., et al., *Effect of Sodium Selenite Administration and Procalcitonin-Guided Therapy on Mortality in Patients With Severe Sepsis or Septic Shock: A Randomized Clinical Trial.* JAMA Intern Med, 2016. **176**(9): p. 1266-76.

5. Feuerecker, M., Sudhoff, L., Crucian, B., Pagel, J.I., Sams, C., Strewe, C., Guo, A., Schelling, G., Briegel, J., Kaufmann, I., Choukèr, A., *Early immune anergy towards recall antigens and mitogens in patients at onset of septic shock.* Sci Rep, 2018. 8(1): p. 1754.

11. References

1. Martin, G.S., *Sepsis, severe sepsis and septic shock: changes in incidence, pathogens and outcomes.* Expert Rev Anti Infect Ther, 2012. **10**(6): p. 701-6.

2. *American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis.* Crit Care Med, 1992. **20**(6): p. 864-74.

3. Levy, M.M., Fink, M.P., Marshall, J.C., Abraham, E., Angus, D., Cook, D., et al., *2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference.* Crit Care Med, 2003. **31**(4): p. 1250-6.

4. Singer, M., Deutschman, C.S., Seymour, C.W., Shankar-Hari, M., Annane, D., Bauer, M., et al., *The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3).* JAMA, 2016. **315**(8): p. 801-10.

5. Hotchkiss, R.S. and Karl, I.E., *The pathophysiology and treatment of sepsis.* N Engl J Med, 2003. **348**(2): p. 138-50.

6. Angus, D.C., Linde-Zwirble, W.T., Lidicker, J., Clermont, G., Carcillo, J., and Pinsky, M.R., *Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care.* Crit Care Med, 2001. **29**(7): p. 1303-10.

7. Martin, G.S., Mannino, D.M., Eaton, S., and Moss, M., *The epidemiology of sepsis in the United States from 1979 through 2000.* N Engl J Med, 2003. **348**(16): p. 1546-54.

8. Danai, P. and Martin, G.S., *Epidemiology of sepsis: recent advances.* Curr Infect Dis Rep, 2005. **7**(5): p. 329-34.

9. Quenot, J.P., Binquet, C., Kara, F., Martinet, O., Ganster, F., Navellou, J.C., et al., *The epidemiology of septic shock in French intensive care units: the prospective multicenter cohort EPISS study.* Crit Care, 2013. **17**(2): p. R65.

10. Sands, K.E., Bates, D.W., Lanken, P.N., Graman, P.S., Hibberd, P.L., Kahn, K.L., et al., *Epidemiology of sepsis syndrome in 8 academic medical centers.* JAMA, 1997. **278**(3): p. 234-40.

11. Dellinger, R.P., Levy, M.M., Rhodes, A., Annane, D., Gerlach, H., Opal, S.M., et al., *Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2012.* Crit Care Med, 2013. **41**(2): p. 580-637.

12. Kumar, A., Roberts, D., Wood, K.E., Light, B., Parrillo, J.E., Sharma, S., et al., *Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock.* Crit Care Med, 2006. **34**(6): p. 1589-96.

13. Reinhart, K., Brunkhorst, F.M., Bone, H.G., Bardutzky, J., Dempfle, C.-E., Forst, H., et al., *Prävention, Diagnose, Nachsorge und Therapie der Sepsis.* 2010. Available from: http://www.awmf.org/uploads/tx_szleitlinien/079-001l_S2k_Sepsis_2010-abgelaufen.pdf [Internet]; Last updated: 01.02.2010, cited: 17.08.2015, archived at: http://www.webcitation.org/6aqqUzwFT.

14. Martins, P.S., Kallas, E.G., Neto, M.C., Dalboni, M.A., Blecher, S., and Salomao, R., *Upregulation of reactive oxygen species generation and phagocytosis, and increased apoptosis in human neutrophils during severe sepsis and septic shock.* Shock, 2003. **20**(3): p. 208-12.

15. Oberholzer, A., Oberholzer, C., and Moldawer, L.L., *Cytokine signaling--regulation of the immune response in normal and critically ill states.* Crit Care Med, 2000. **28**(4 Suppl): p. N3-12.

16. Abbas, A.K., Murphy, K.M., and Sher, A., *Functional diversity of helper T lymphocytes.* Nature, 1996. **383**(6603): p. 787-93.

17. Stearns-Kurosawa, D.J., Osuchowski, M.F., Valentine, C., Kurosawa, S., and Remick, D.G., *The pathogenesis of sepsis.* Annu Rev Pathol, 2011. **6**: p. 19-48.

18. Rittirsch, D., Flierl, M.A., and Ward, P.A., *Harmful molecular mechanisms in sepsis.* Nat Rev Immunol, 2008. **8**(10): p. 776-87.

19. Fink, M.P., *Reactive oxygen species as mediators of organ dysfunction caused by sepsis, acute respiratory distress syndrome, or hemorrhagic shock: potential benefits of resuscitation with Ringer's ethyl pyruvate solution.* Curr Opin Clin Nutr Metab Care, 2002. **5**(2): p. 167-74.

20. Wiersinga, W.J., *Current insights in sepsis: from pathogenesis to new treatment targets.* Curr Opin Crit Care, 2011. **17**(5): p. 480-6.

21. Ertel, W., Keel, M., Neidhardt, R., Steckholzer, U., Kremer, J.P., Ungethuem, U., et al., *Inhibition of the defense system stimulating interleukin-12 interferon-gamma pathway during critical Illness.* Blood, 1997. **89**(5): p. 1612-20.

22. Hotchkiss, R.S., Swanson, P.E., Freeman, B.D., Tinsley, K.W., Cobb, J.P., Matuschak, G.M., et al., *Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction.* Crit Care Med, 1999. **27**(7): p. 1230-51.

23. Osuchowski, M.F., Craciun, F., Weixelbaumer, K.M., Duffy, E.R., and Remick, D.G., *Sepsis chronically in MARS: systemic cytokine responses are always mixed regardless of the outcome, magnitude, or phase of sepsis.* J Immunol, 2012. **189**(9): p. 4648-56.

24. Huttunen, R. and Aittoniemi, J., *New concepts in the pathogenesis, diagnosis and treatment of bacteremia and sepsis.* J Infect, 2011. **63**(6): p. 407-19.

25. *Placebo Controlled Trial of Sodium Selenite and Procalcitonin Guided Antimicrobial Therapy in Severe Sepsis (SISPCT).* 2009. Available from: https://www.clinicaltrials.gov/show/NCT00832039 [Internet]; Last updated: 28.06.2013, cited: 21.09.2015, archived at: http://www.webcitation.org/6aqxI6YMs.

26. *Mitteilung der Ständigen Impfkommission am Robert Koch-Institut (RKI).* Epidemiologisches Bulletin, 2015. **34/2015** (Empfehlungen der Ständigen Impfkommission (STIKO) am Robert Koch-Institut).

27. Suzuki, N. and Sakane, T., *Mechanism of T cell-derived helper factor production upon stimulation with pokeweed mitogen in humans.* Clin Exp Immunol, 1988. **71**(2): p. 343-9.

28. Waldmann, T.A. and Broder, S., *Polyclonal B-cell activators in the study of the regulation of immunoglobulin synthesis in the human system.* Adv Immunol, 1982. **32**: p. 1-63.

29. Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., et al., *Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene.* Science, 1998. **282**(5396): p. 2085-8.

30. Nishihira, J. and O'Flaherty, J.T., *Phorbol myristate acetate receptors in human polymorphonuclear neutrophils.* J Immunol, 1985. **135**(5): p. 3439-47.

31. Iliodromitis, E.K., Miki, T., Liu, G.S., Downey, J.M., Cohen, M.V., and Kremastinos, D.T., *The PKC activator PMA preconditions rabbit heart in the presence of adenosine receptor blockade: is 5'-nucleotidase important?* J Mol Cell Cardiol, 1998. **30**(11): p. 2201-11.

32. Touraine, J.L., Hadden, J.W., Touraine, F., Hadden, E.M., Estensen, R., and Good, R.A., *Phorbol myristate acetate: a mitogen selective for a T-lymphocyte subpopulation.* J Exp Med, 1977. **145**(2): p. 460-5.

33. Pecivova, J., Macickova, T., Svitekova, K., and Nosal, R., *Quercetin inhibits degranulation and superoxide generation in PMA stimulated neutrophils.* Interdiscip Toxicol, 2012. **5**(2): p. 81-3.

34. Gabriel, H., Muller, H.J., Urhausen, A., and Kindermann, W., *Suppressed PMA-induced oxidative burst and unimpaired phagocytosis of circulating granulocytes one week after a long endurance*

exercise. Int J Sports Med, 1994. **15**(7): p. 441-5.

35. Fabiani, R., De Bartolomeo, A., Rosignoli, P., and Morozzi, G., *Antioxidants prevent the lymphocyte DNA damage induced by PMA-stimulated monocytes.* Nutr Cancer, 2001. **39**(2): p. 284-91.

36. *Dynabeads® Human T-Activator CD3/CD28 - for physiological activation of human T cells.* Available from: http://www.lifetechnologies.com/de/de/home/references/protocols/proteins-expression-isolation-and-analysis/t-cell-activation-and-expansion/dynabeads-human-t-activator-cd3-cd28.html#prot1 [Internet]; Last updated: 05.05.2009, cited: 21.09.2015, archived at: http://www.webcitation.org/6aqx2fmiV.

37. Kaufmann, S., *Basiswissen Immunologie*. 7th ed. Berlin: Springer Medizin; 2012.

38. de Jager, W., te Velthuis, H., Prakken, B.J., Kuis, W., and Rijkers, G.T., *Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells.* Clin Diagn Lab Immunol, 2003. **10**(1): p. 133-9.

39. Crucian, B., Stowe, R.P., Mehta, S., Quiriarte, H., Pierson, D., and Sams, C., *Alterations in adaptive immunity persist during long-duration spaceflight.* NPJ Microgravity, 2015. **1**: p. 15013.

40. Le Gall, J.R., Lemeshow, S., and Saulnier, F., *A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study.* JAMA, 1993. **270**(24): p. 2957-63.

41. Knaus, W.A., Draper, E.A., Wagner, D.P., and Zimmerman, J.E., *APACHE II: a severity of disease classification system.* Crit Care Med, 1985. **13**(10): p. 818-29.

42. Bloos, F., Trips, E., Nierhaus, A., Briegel, J., Heyland, D.K., Jaschinski, U., et al., *Effect of Sodium Selenite Administration and Procalcitonin-Guided Therapy on Mortality in Patients With Severe Sepsis or Septic Shock: A Randomized Clinical Trial.* JAMA Intern Med, 2016. **176**(9): p. 1266-76.

43. Holländer, G.A. and Barthlott, T., *Immunologie: Grundlagen für Klinik und Praxis*. 1st ed. München: Elsevier, Urban & Fischer; 2006.

44. Ridker, P.M., Rifai, N., Stampfer, M.J., and Hennekens, C.H., *Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men.* Circulation, 2000. **101**(15): p. 1767-72.

45. Thiel, M., Chouker, A., Ohta, A., Jackson, E., Caldwell, C., Smith, P., et al., *Oxygenation inhibits the physiological tissue-protecting mechanism and thereby exacerbates acute inflammatory lung injury.* PLoS Biol, 2005. **3**(6): p. e174.

46. Chouker, A., Thiel, M., Lukashev, D., Ward, J.M., Kaufmann, I., Apasov, S., et al., *Critical role of hypoxia and A2A adenosine receptors in liver tissue-protecting physiological anti-inflammatory pathway.* Mol Med, 2008. **14**(3-4): p. 116-23.

47. Oppert, M., Engel, C., Brunkhorst, F.M., Bogatsch, H., Reinhart, K., Frei, U., et al., *Acute renal failure in patients with severe sepsis and septic shock--a significant independent risk factor for mortality: results from the German Prevalence Study.* Nephrol Dial Transplant, 2008. **23**(3): p. 904-9.

48. Tamayo, E., Fernandez, A., Almansa, R., Carrasco, E., Heredia, M., Lajo, C., et al., *Pro- and anti-inflammatory responses are regulated simultaneously from the first moments of septic shock.* Eur Cytokine Netw, 2011. **22**(2): p. 82-7.

49. Castell, J.V., Geiger, T., Gross, V., Andus, T., Walter, E., Hirano, T., et al., *Plasma clearance, organ distribution and target cells of interleukin-6/hepatocyte-stimulating factor in the rat.* Eur J Biochem, 1988. **177**(2): p. 357-61.

50. Volk, H.D., Reinke, P., and Docke, W.D., *Clinical aspects: from systemic inflammation to 'immunoparalysis'.* Chem Immunol, 2000. **74**: p. 162-77.

51. Van Snick, J., *Interleukin-6: an overview.* Annu Rev Immunol, 1990. **8**: p. 253-78.

52. Trappe, U. and Riess, H., *[Basics in the pathophysiology of sepsis].* Hamostaseologie, 2005. **25**(2): p. 175-82.

53. Novotny, A.R., Reim, D., Assfalg, V., Altmayr, F., Friess, H.M., Emmanuel, K., et al., *Mixed antagonist response and sepsis severity-dependent dysbalance of pro- and anti-inflammatory responses at the onset of postoperative sepsis.* Immunobiology, 2012. **217**(6): p. 616-21.

54. Hotchkiss, R.S., Osmon, S.B., Chang, K.C., Wagner, T.H., Coopersmith, C.M., and Karl, I.E., *Accelerated lymphocyte death in sepsis occurs by both the death receptor and mitochondrial pathways.* J Immunol, 2005. **174**(8): p. 5110-8.

55. Chang, K.C., Unsinger, J., Davis, C.G., Schwulst, S.J., Muenzer, J.T., Strasser, A., et al., *Multiple triggers of cell death in sepsis: death receptor and mitochondrial-mediated apoptosis.* FASEB J, 2007. **21**(3): p. 708-19.

56. Kasten, K.R., Tschop, J., Adediran, S.G., Hildeman, D.A., and Caldwell, C.C., *T cells are potent early mediators of the host response to sepsis.* Shock, 2010. **34**(4): p. 327-36.

57. Hotchkiss, R.S., Coopersmith, C.M., McDunn, J.E., and Ferguson, T.A., *The sepsis seesaw: tilting toward immunosuppression.* Nat Med, 2009. **15**(5): p. 496-7.

58. Hermann, C., von Aulock, S., Graf, K., and Hartung, T., *A model of human whole blood lymphokine release for in vitro and ex vivo use.* J Immunol Methods, 2003. **275**(1-2): p. 69-79.

59. Saka, G., Kreke, J.E., Schaefer, A.J., Chang, C.C., Roberts, M.S., Angus, D.C., et al., *Use of dynamic microsimulation to predict disease progression in patients with pneumonia-related sepsis.* Crit Care, 2007. **11**(3): p. R65.

60. Remick, D.G., Newcomb, D.E., Bolgos, G.L., and Call, D.R., *Comparison of the mortality and inflammatory response of two models of sepsis: lipopolysaccharide vs. cecal ligation and puncture.* Shock, 2000. **13**(2): p. 110-6.

61. Frazier, W.J. and Hall, M.W., *Immunoparalysis and Adverse Outcomes from Critical Illness.* Pediatric clinics of North America, 2008. **55**(3): p. 647-xi.

62. Sompayrac, L., *How the immune system works*. 4th ed. Chichester, West-Sussex; Hoboken, NJ: Wiley-Blackwell; 2012.

63. Becze, Z., Molnar, Z., and Fazakas, J., *Can procalcitonin levels indicate the need for adjunctive therapies in sepsis?* Int J Antimicrob Agents, 2015. **46 Suppl 1**: p. S13-8.

64. Zhang, Q., Raoof, M., Chen, Y., Sumi, Y., Sursal, T., Junger, W., et al., *Circulating mitochondrial DAMPs cause inflammatory responses to injury.* Nature, 2010. **464**(7285): p. 104-7.

65. Karow, T. and Lang-Roth, R., *Allgemeine und spezielle Pharmakologie und Toxikologie 2007*. 15. Auflage. Pulheim: Thomas Karow; 2006.

66. Nowak, M., Lynch, L., Yue, S., Ohta, A., Sitkovsky, M., Balk, S.P., et al., *The A2aR adenosine receptor controls cytokine production in iNKT cells.* Eur J Immunol, 2010. **40**(3): p. 682-7.

67. Linden, J., *Adenosine in tissue protection and tissue regeneration.* Mol Pharmacol, 2005. **67**(5): p. 1385-7.

68. Abraham, E., Wunderink, R., Silverman, H., Perl, T.M., Nasraway, S., Levy, H., et al., *Efficacy and safety of monoclonal antibody to human tumor necrosis factor alpha in patients with sepsis syndrome. A randomized, controlled, double-blind, multicenter clinical trial. TNF-alpha MAb Sepsis Study Group.* JAMA, 1995. **273**(12): p. 934-41.

69. Hinz, B., Jauch, O., Noky, T., Friesecke, S., Abel, P., and Kaiser, R., *CytoSorb, a novel therapeutic approach for patients with septic shock: a case report.* Int J Artif Organs, 2015. **38**(8): p. 461-4.

70. Meisel, C., Schefold, J.C., Pschowski, R., Baumann, T., Hetzger, K., Gregor, J., et al., *Granulocyte-macrophage colony-stimulating factor to reverse sepsis-associated immunosuppression: a double-blind, randomized, placebo-controlled multicenter trial.* Am J Respir Crit Care Med, 2009. **180**(7): p. 640-8.

71. Docke, W.D., Randow, F., Syrbe, U., Krausch, D., Asadullah, K., Reinke, P., et al., *Monocyte deactivation in septic patients: restoration by IFN-gamma treatment.* Nat Med, 1997. **3**(6): p. 678-81.

72. Nierhaus, A., Montag, B., Timmler, N., Frings, D.P., Gutensohn, K., Jung, R., et al., *Reversal of immunoparalysis by recombinant human granulocyte-macrophage colony-stimulating factor in patients with severe sepsis.* Intensive Care Med, 2003. **29**(4): p. 646-51.

73. Limaye, A.P., Kirby, K.A., Rubenfeld, G.D., Leisenring, W.M., Bulger, E.M., Neff, M.J., et al., *Cytomegalovirus reactivation in critically ill immunocompetent patients.* JAMA, 2008. **300**(4): p. 413-22.

74. Luyt, C.E., Combes, A., Deback, C., Aubriot-Lorton, M.H., Nieszkowska, A., Trouillet, J.L., et al., *Herpes simplex virus lung infection in patients undergoing prolonged mechanical ventilation.* Am J Respir Crit Care Med, 2007. **175**(9): p. 935-42.

75. Opal, S.M., *New perspectives on immunomodulatory therapy for bacteraemia and sepsis.* Int J Antimicrob Agents, 2010. **36 Suppl 2**: p. S70-3.

76. Huang, G.K., Stewardson, A.J., and Grayson, M.L., *Back to basics: hand hygiene and isolation.* Curr Opin Infect Dis, 2014. **27**(4): p. 379-89.

77. Bone, R.C., Fisher, C.J., Jr., Clemmer, T.P., Slotman, G.J., Metz, C.A., and Balk, R.A., *A controlled clinical trial of high-dose methylprednisolone in the treatment of severe sepsis and septic shock.* N Engl J Med, 1987. **317**(11): p. 653-8.

78. Annane, D., Bellissant, E., Bollaert, P.E., Briegel, J., Confalonieri, M., De Gaudio, R., et al., *Corticosteroids in the treatment of severe sepsis and septic shock in adults: a systematic review.* JAMA, 2009. **301**(22): p. 2362-75.

79. Annane, D., Bellissant, E., Bollaert, P.E., Briegel, J., Keh, D., and Kupfer, Y., *Corticosteroids for treating sepsis.* Cochrane Database Syst Rev, 2015. http://cochranelibrary-wiley.com/doi/10.1002/14651858.CD002243.pub3/full DOI:10.1002/14651858.CD002243.pub3(12): p. CD002243.

80. Sprung, C.L., Annane, D., Keh, D., Moreno, R., Singer, M., Freivogel, K., et al., *Hydrocortisone therapy for patients with septic shock.* N Engl J Med, 2008. **358**(2): p. 111-24.

81. Bo, L., Wang, F., Zhu, J., Li, J., and Deng, X., *Granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) for sepsis: a meta-analysis.* Crit Care, 2011. **15**(1): p. R58.

82. Schefold, J.C., *Immunostimulation using granulocyte- and granulocyte-macrophage colony stimulating factor in patients with severe sepsis and septic shock.* Crit Care, 2011. **15**(2): p. 136.

83. Mathias, B., Szpila, B.E., Moore, F.A., Efron, P.A., and Moldawer, L.L., *A Review of GM-CSF Therapy in Sepsis.* Medicine (Baltimore), 2015. **94**(50): p. e2044.

84. Venet, F., Filipe-Santos, O., Lepape, A., Malcus, C., Poitevin-Later, F., Grives, A., et al., *Decreased T-cell repertoire diversity in sepsis: a preliminary study.* Crit Care Med, 2013. **41**(1): p. 111-9.

85. Englert, J.A. and Fink, M.P., *The multiple organ dysfunction syndrome and late-phase mortality in sepsis.* Curr Infect Dis Rep, 2005. **7**(5): p. 335-41.

86. Monneret, G., Venet, F., Pachot, A., and Lepape, A., *Monitoring immune dysfunctions in the septic patient: a new skin for the old ceremony.* Mol Med, 2008. **14**(1-2): p. 64-78.

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13. Affidavit

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

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