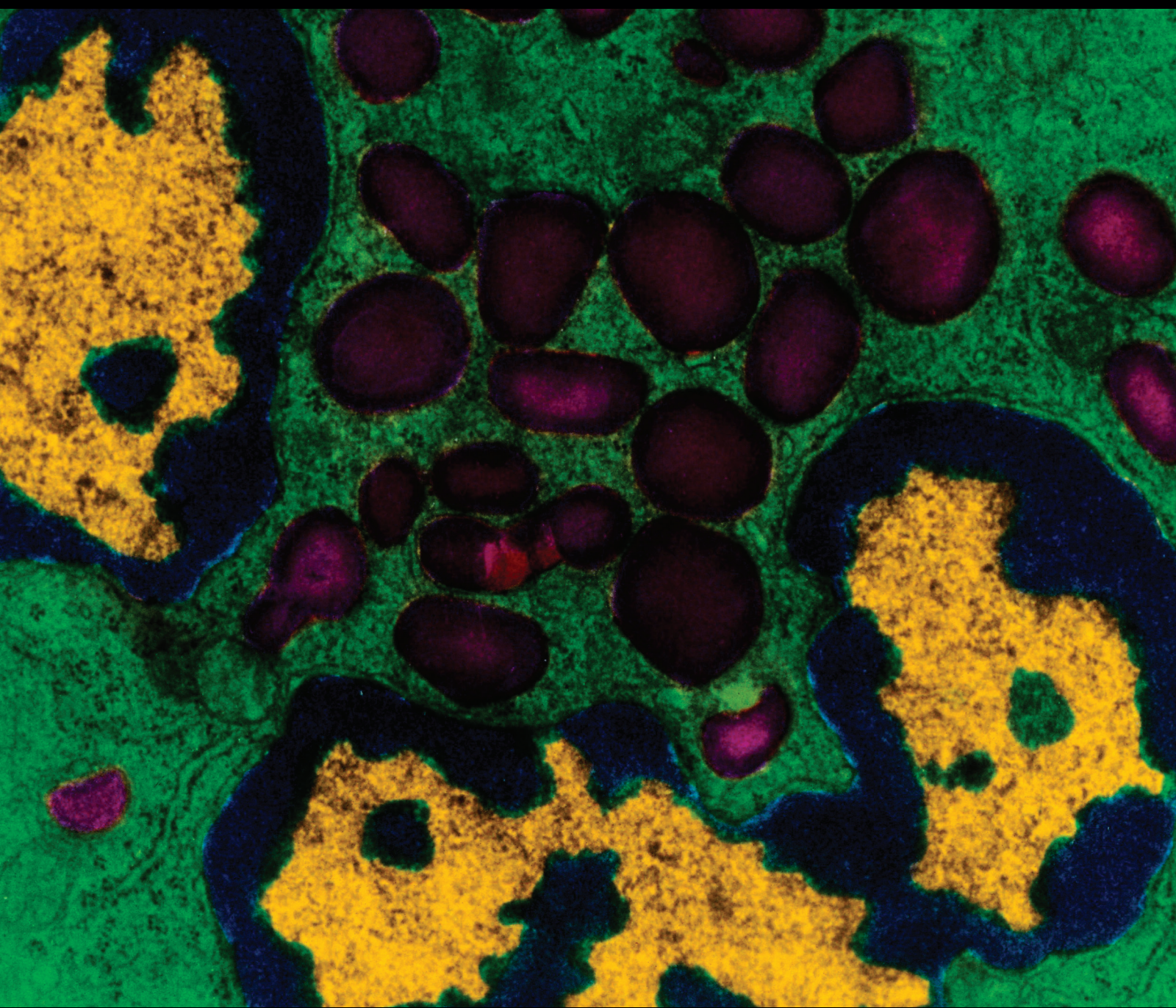
Mediators of Inflammation

Immune Response in Critically Ill Patients

Lead Guest Editor: Maja Surbatovic
Guest Editors: Danilo Vojvodic and Wasim S. Khan



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Editorial

Immune Response in Critically Ill Patients

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Critical illness is defined by presence of altered organ function in acutely ill patients such that homeostasis cannot be maintained without medical intervention in intensive care units, such as mechanical ventilation, vasoactive support for hemodynamics, renal replacement therapy, and so on. It usually involves two or more organ systems. Immune dysfunction is common in critically ill patients and it may modulate immune response and affect patient morbidity and mortality, particularly in severe trauma and/or sepsis. Immune cells and mediators, in critical care setting, are understudied and do represent challenging area. Better understanding of potential beneficial and/or harmful effects of pro- and/or anti-inflammatory response will improve process of intensive care. Potential therapeutic interventions may improve clinical outcome. Pro- and anti-inflammatory mediators can be predictive biomarkers of organ dysfunction and outcome in critically ill patients also [1]. Immune cells' and mediators' role in immune response in critical illness is not yet fully elucidated. Also, their role as predictive biomarkers of organ dysfunction and outcome is of interest for both researchers and clinicians. Profound knowledge of this complex conundrum is essential for potential therapeutic procedures regarding immune dysfunction in critically ill patients.

Source of sepsis is important component influencing both biomarkers in systemic circulation and therapeutic options regarding source control [2]. In this special issue, M. Holub et al. focused their research on correlation of selected biomarkers with origin and severity of sepsis. They analyzed the plasma levels of cytokines (IL-6, IL-8, and IL-10), chemokines (CCL2/MCP-1, MIP-1β), heparin-binding protein (HBP), soluble CD14 (sCD14), and cortisol in the blood from septic patients obtained during the first 96 hours of intensive care unit hospitalization. The authors showed that plasma concentrations of MCP-1, sCD14, IL-6, and IL-10 were significantly higher in patients with community-acquired pneumonia and infective endocarditis compared to those with bacterial meningitis. Their findings suggest that MCP-1, sCD14, IL-6, IL-10, cortisol, and HBP are modulated by the source of sepsis and that elevated MCP-1 and cortisol plasma levels are associated with sepsis-induced organ dysfunction.

The cholinergic system plays an important role in maintaining and modulating an adequate immune response upon an inflammatory episode. Cholinergic activity, acting extrasynaptically (nonneuronal acetylcholine), has been proposed to be a major mediator of the neuroimmune response to inflammation in a classical feedback reflex manner [3]. Serum cholinesterase (BChE), an enzyme that hydrolyzes acetylcholine, plays an important role during inflammatory response and serves as an accurate index of cholinergic activity. In this special issue, A. Zivkovic et al. investigated BChE activity in septic patients using a point-of-care system. Levels of conventional inflammatory markers (C-reactive protein, procalcitonin, IL-4, IL-6, IL-10, TNF-α, and midrange-proadrenomedullin—MR-proADM) were also measured. The authors observed a strong, sustained reduction in BChE activity in nonsurvivors as compared to

survivors in a 90-day observation period. Reduced BChE activity when measured at the ICU admission effectively differentiated between the 90-day survivor and the nonsurvivor patient groups. They estimated a critical BChE level of 1.661 kU/l (CI 0.5–0.8, 94% sensitivity, 48% specificity, and AUC 0.7) to best predict patient outcome.

It is difficult to find adequate biomarker of immune response in critical illness, regardless of its cause, with good predictive value regarding outcome because there is wide and complex array of immune-related mediators. Many of them were explored in this clinical setting with contradictory results. Recently, some readily available parameters, originated from routine complete blood count (CBC), have been investigated as potential biomarkers [4, 5]. In this special issue, Surbatovic et al. assessed prognostic value of neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR), platelet-to-lymphocyte ratio (PLR), and mean platelet volume-to-platelet count (MPV/PC) ratio regarding outcome in total of 392 critically ill patients with secondary sepsis and/or trauma. Patients were divided into four subgroups: peritonitis, pancreatitis, trauma with sepsis, and trauma without sepsis. The authors demonstrated that NLR and MPV/PC levels were significantly higher in nonsurvivors (AUC/ROC of 0.681 and 0.592, respectively, in peritonitis subgroup; 0.717 and 0.753, respectively, in pancreatitis subgroup); MLR and PLR did not differ significantly. There was no significant difference of investigated biomarkers between survivors and nonsurvivors in trauma patients with and without sepsis except for PLR in trauma without sepsis subgroup (significantly higher in nonsurvivors; AUC/ROC of 0.719). Independent predictor of lethal outcome was NLR in whole cohort and in peritonitis subgroup as well as MPV in pancreatitis subgroup. Also, there were statistically significant differences in MPV/PC, MLR, and PLR values regarding nature of bacteremia. In general, the lowest levels had patients with Gram-positive blood cultures. The authors concluded that NLR and MPV were very good independent predictors of lethal outcome. For the first time, they demonstrated that nature of bacteremia influences MPV/PC, MLR, and PLR.

Neutrophils are one of the first lines of defense against invading pathogens and are responsible for containing and eliminating invading pathogens. Neutrophils are multifaceted innate immune cells that also modulate the inflammatory response and initiate the adaptive immune responses to sepsis via the release of cytokines [6]. Following activation, triggered either by frustrated phagocytosis or sustained inflammation, neutrophils release neutrophil extracellular traps (NETs), whereby nuclear DNA laden with histones and granular contents are liberated into the extracellular space, which trap and kill extracellular bacteria [7]. In this special issue, J. Patel et al. presented a prospective observational cohort study enrolling 21 healthy age-matched controls and 39 sepsis and 60 severe sepsis patients from acute admissions to two UK hospitals. Patients had sequential bloods for the ex vivo assessment of NETosis in response to phorbol myristate acetate (PMA) using a fluorometric technique and chemotaxis using time-lapse video microscopy. The authors demonstrated that ex vivo NETosis was significantly reduced in patients with severe sepsis, compared to patients with sepsis and controls. PMA NETosis from patients with septic shock was significantly reduced further compared to controls. Reduced NETosis at baseline was significantly associated with an increased 30-day and 90-day mortality in sepsis patients. These findings were accompanied by defects in neutrophil migration and delayed apoptosis. Resolution of sepsis was not associated with the return to baseline levels of NETosis or migration. The authors concluded that sepsis induces significant changes in neutrophil function with the degree of dysfunction corresponding to the severity of the septic insult which persists beyond physiological recovery from sepsis. The changes induced lead to the failure to effectively contain and eliminate the invading pathogens and contribute to sepsis-induced immunosuppression. For the first time, they demonstrated that reduced ex vivo NETosis is associated with poorer outcomes from sepsis.

Sepsis progression and septic liver dysfunction are characterized by complex pathophysiological alterations, including processes like reactive oxygen species (ROS) and nitrogen species (RNS) release, inflammation, and apoptosis [8]. In this special issue, Z. Jiang et al. used a lipopolysaccharide (LPS) stimulation model to simulate the septic liver injury and to investigate the effect of Sophora alkaloid sophocarpine on LPS-stimulated mice with endotoxemia. The authors demonstrated that sophocarpine increases the survival rate of mice and attenuates the LPS-induced liver injury, which is indicated by pathology and serum liver enzymes. Further research found that sophocarpine ameliorated hepatic oxidative stress indicators (H_2O_2, O_2^-, and NO) and enhanced the expression of antioxidant molecules such as superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH). In addition, sophocarpine also attenuated regional and systematic inflammation and further reduced apoptosis of hepatocytes. Mechanistic evidence was also investigated in this study as sophocarpine inhibited hepatic expression of the CYP2E/Nrf2 pathway during oxidative stress, inactivated p38/JNK cascade and NF-κB pathway, and suppressed PI3K/AKT signaling that reduced apoptosis. The authors concluded that their investigation unveiled the protective role of sophocarpine in LPS-stimulated oxidative reaction, inflammation, and apoptosis suggesting its promising role in attenuating inflammation and liver injury in septic endotoxemia.

Trauma has profound effect on immune response and may lead to diminished functionality of monocytes and subsequently their IL-1β release. IL-1β plays an important role in host immunity and protection against infections. Its biological activation via IL-1β precursor processing requires the transcription of inflammasome components and their activation. Activity of NOD-like receptor inflammasomes- (NLR-) like NLRP3 leads to the maturation of IL-1β [9]. In this special issue, S. Kany et al. investigated the role of the NLRP3 inflammasome in impaired monocyte functionality after a traumatic injury. Ex vivo-in vitro stimulation of isolated $CD14^+$ monocytes with lipopolysaccharide (LPS)

showed a significantly higher IL-1β secretion in healthy volunteers compared to trauma patients after admission. Reduced IL-1β secretion was paralleled by significantly lowered gene expression of NLRP3 in monocytes from trauma patients compared to those of healthy volunteers. Transfection of monocytes with NLRP3-encoding plasmid recovered the functionality of monocytes from trauma patients regarding the IL-1β secretion. The authors concluded that $CD14^+$ monocytes from trauma patients are significantly diminished in their function, and that the presence of NLRP3 components is necessary in recovering the ability of monocytes to produce active IL-1β. Also, this recovery of the NLRP3 inflammasome in monocytes may imply a new target for treatment and therapy of immune suppression after severe trauma.

Finally, immune response is important component in very specific setting of out-of-hospital cardiac arrest (OHCA). Survival of OHCA is affected by global ischaemia followed by a pronounced reperfusion injury due to return of spontaneous circulation (ROSC). The ischaemia-reperfusion response induces a complex activation of immunologic pathways leading to subsequent systemic inflammatory immune reaction. This phenomenon is known as the post-cardiac arrest syndrome (PCAS). Induction of therapeutic hypothermia (TH) in this setting also influences immunoinflammatory response [10]. In this special issue, M. Braunstein et al. evaluated immediate immunological changes following cardiopulmonary resuscitation (CPR). mRNA expression levels of selected immunomodulatory cytokines in OHCA survivors were detected and correlated to clinical parameters. OHCA survivors with sustained unconsciousness after return of spontaneous circulation (ROSC) were included. PAXgene whole blood samples were drawn immediately after initiation of CPR and subsequently after 6 h, 12 h, 24 h, 48 h, and 72 h. TNF-alpha, IL-8, IL-10, and IL-1ra mRNA levels were quantified by RT-qPCR and compared to multiple organ failure, a 30-day survival, and the induction of TH. The authors demonstrated characteristic time-dependent cytokine profile in the early postresuscitation period. High initial TNF-alpha and IL-8 mRNA levels were followed by a significant decrease. IL-1ra mRNA levels significantly increased beginning after 6 h. Nonsurvivors showed significantly higher IL-8 mRNA levels immediately after CPR. TH induced significantly higher IL-1ra mRNA levels compared to normothermia. The authors concluded that significant mRNA cytokine expression changes are already detectable immediately after initiation of CPR. These expressional changes are significantly different depending on a 30-day survival. Also, TH seems to attenuate proinflammatory immune reaction by a significant increase of IL-1ra mRNA levels.

Current special issue provides broad spectrum of interesting topics in the field of immune response in critically ill. Hopefully, it will be useful to both clinicians and researchers in this exciting field.

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References

[1] M. Surbatovic, M. Veljovic, J. Jevdjic, N. Popovic, D. Djordjevic, and S. Radakovic, "Immunoinflammatory response in critically ill patients: severe sepsis and/or trauma," *Mediators of Inflammation*, vol. 2013, Article ID 362793, 11 pages, 2013.

[2] J. Rello, F. Valenzuela-Sánchez, M. Ruiz-Rodriguez, and S. Moyano, "Sepsis: a review of advances in management," *Advances in Therapy*, vol. 34, no. 11, pp. 2393–2411, 2017.

[3] U. Andersson and K. J. Tracey, "Neural reflexes in inflammation and immunity," *Journal of Experimental Medicine*, vol. 209, no. 6, pp. 1057–1068, 2012.

[4] X. Liu, Y. Shen, H. Wang, Q. Ge, A. Fei, and S. Pan, "Prognostic significance of neutrophil-to-lymphocyte ratio in patients with sepsis: a prospective observational study," *Mediators of Inflammation*, vol. 2016, Article ID 8191254, 8 pages, 2016.

[5] G. H. Oh, S. P. Chung, Y. S. Park et al., "Mean platelet volume to platelet count ratio as a promising predictor of early mortality in severe sepsis," *Shock*, vol. 47, no. 3, pp. 323–330, 2017.

[6] P. H. C. Leliefeld, C. M. Wessels, L. P. H. Leenen, L. Koenderman, and J. Pillay, "The role of neutrophils in immune dysfunction during severe inflammation," *Critical Care*, vol. 20, no. 1, p. 73, 2016.

[7] M. Hashiba, A. Huq, A. Tomino et al., "Neutrophil extracellular traps in patients with sepsis," *The Journal of Surgical Research*, vol. 194, no. 1, pp. 248–254, 2015.

[8] Y. Hattori, K. Hattori, T. Suzuki, and N. Matsuda, "Recent advances in the pathophysiology and molecular basis of sepsis-associated organ dysfunction: novel therapeutic implications and challenges," *Pharmacology & Therapeutics*, vol. 177, pp. 56–66, 2017.

[9] Y. He, H. Hara, and G. Nunez, "Mechanism and regulation of NLRP3 inflammasome activation," *Trends in Biochemical Sciences*, vol. 41, no. 12, pp. 1012–1021, 2016.

[10] J. Bro-Jeppesen, J. Kjaergaard, P. Stammet et al., "Predictive value of interleukin-6 in post-cardiac arrest patients treated with targeted temperature management at 33°C or 36°C," *Resuscitation*, vol. 98, pp. 1–8, 2016.

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Research Article

Neutrophil-to-Lymphocyte Ratio, Monocyte-to-Lymphocyte Ratio, Platelet-to-Lymphocyte Ratio, and Mean Platelet Volume-to-Platelet Count Ratio as Biomarkers in Critically Ill and Injured Patients: Which Ratio to Choose to Predict Outcome and Nature of Bacteremia?

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Background. Neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR), platelet-to-lymphocyte ratio (PLR), and mean platelet volume-to-platelet count (MPV/PC) ratio are readily available parameters that might have discriminative power regarding outcome. The aim of our study was to assess prognostic value of these biomarkers regarding outcome in critically ill patients with secondary sepsis and/or trauma. *Methods*. A total of 392 critically ill and injured patients, admitted to surgical ICU, were enrolled in a prospective observational study. Leukocyte and platelet counts were recorded upon fulfilling Sepsis-3 criteria and for traumatized Injury Severity Score > 25 points. Patients were divided into four subgroups: peritonitis, pancreatitis, trauma with sepsis, and trauma without sepsis. *Results*. NLR and MPV/PC levels were significantly higher in nonsurvivors (AUC/ROC of 0.681 and 0.592, resp., in the peritonitis subgroup; 0.717 and 0.753, resp., in the pancreatitis subgroup); MLR and PLR did not differ significantly. There was no significant difference of investigated biomarkers between survivors and nonsurvivors in trauma patients with and without sepsis except for PLR in the trauma without sepsis subgroup (significantly higher in nonsurvivors, AUC/ROC of 0.719). Independent predictor of lethal outcome was NLR in the whole cohort and in the peritonitis subgroup as well as MPV in the pancreatitis subgroup. Also, there were statistically significant differences in MPV/PC, MLR, and PLR values regarding nature of bacteremia. In general, the lowest levels had been found in patients with Gram-positive blood cultures. *Conclusions*. NLR and MPV were very good independent predictors of lethal outcome. For the first time, we demonstrate that nature of bacteremia influences MPV/PC, MLR, and PLR. In heterogeneous cohort subgroup, analysis is essential.

1. Introduction

In the treatment of critically ill and/or injured patients, it is important to detect those who are at high risk for lethal outcome. Major determinant of outcome is intensity of insult (infection, trauma) as well as immunoinflammatory response [1, 2]. It is difficult to find adequate biomarker of immune response in critical illness, regardless of its cause, with good predictive value regarding outcome because there is wide and complex array of immune-related mediators. Many of them were explored in this clinical setting with contradictory results. Recently, some readily available parameters, originated from routine complete blood count (CBC), have been investigated as potential biomarkers with mixed results and no consensus so far regarding its accuracy and clinical usefulness: neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR), platelet-to-lymphocyte ratio (PLR), and mean platelet volume-to-platelet count (MPV/PC) ratio [3–6].

Bearing in mind how intertwined immune and coagulation cascades are, the aim of our prospective observational study was to assess the prognostic value of NLR, MLR, PLR, and MPV/PC ratio regarding outcome in a cohort of critically ill patients with secondary sepsis and/or trauma. Outcome measure was hospital mortality. Our secondary endpoint was to assess possible differences of these biomarkers regarding different blood cultures in patients with documented bacteremia.

2. Material and Methods

2.1. Study Design. A total of 392 critically ill and injured patients, admitted to surgical intensive care unit (SICU), were enrolled in a prospective study conducted in a tertiary university hospital (Military Medical Academy, Belgrade, Serbia) during a 4-year period. Approval in concordance with the Declaration of Helsinki was obtained from local ethics committee as well as informed consent from a patient or first-degree relative. The study was conducted in accordance with the approved guidelines. Patients with secondary sepsis (underlying conditions were peritonitis, pancreatitis, and trauma) were enrolled if they had fulfilled current Sepsis-3 diagnostic criteria for sepsis (formerly severe sepsis) and/or septic shock (acute change in total SOFA score ≥ 2 points and vasopressors required to maintain mean arterial pressure ≥ 65 mmHg and serum lactate level > 2 mmol/L despite adequate volume resuscitation) [7]. The diagnostic criteria encompass any of the following variables thought to be a result of the infection: sepsis-induced hypotension, lactate levels greater than 2 mmol/L, urine output less than 0.5 mL/kg/hr for more than two hours despite adequate fluid resuscitation, acute lung injury with PaO_2/FiO_2 less than 250, creatinine greater than 2.0 mg/dL (176.8 micromol/L), bilirubin greater than 2.0 mg/dL (34.2 micromol/L), platelet count less than 100,000, and coagulopathy (international normalised ratio, INR) greater than 1.5. Also, critically ill patients with severe trauma (Injury Severity Score (ISS) (determined using Abbreviated Injury Scale (AIS)) > 25 points) were enrolled. Only adult patients, age of at least 18 years, were recruited. The exclusion criteria were as follows: (1) secondary sepsis and/or septic shock with an underlying condition other than severe peritonitis, pancreatitis, or trauma, (2) malignant disease of any origin, (3) long-term SICU stay before criteria fulfilment, and (4) preexisting immunodeficiency. All trauma patients were further classified into two subgroups: those who developed secondary sepsis (trauma + sepsis subgroup) and those who did not (trauma subgroup). We sought to recruit four homogenous subgroups in a cohort of critically ill and injured patients: peritonitis subgroup, pancreatitis subgroup, trauma + sepsis subgroup (these three subgroups of patients developed secondary sepsis), and trauma subgroup.

2.2. Blood Measurement. Patient's venous blood was drawn by trained, qualified phlebotomists. If several measurements were available on day of enrollment in the study for some patients, the first one at the time point of diagnostic criteria fulfillment was always used to maximize consistency among patient population. The blood samples were taken into BD Vacutainer K_2 EDTA tubes and were analyzed within 2 hours from venepuncture. A complete blood count was determined by the Siemens Advia 120 hematology system, which is a flow cytometry-based system.

Differentiation of white blood cells is done by the peroxidase and basophil channel. The peroxidase method is a primary differential method on the Advia 120. Peroxidase in the granules of the white blood cells reacts with hydrogen peroxide from reagent and forms dark precipitates within the cells. After measuring the light scatter which represents the size of the cell and absorption showing the level of staining, the analyzer defines next cell population: neutrophils, monocytes, eosinophils, and large unstained cells, while lymphocytes and basophils appear as one cluster. This requires a further method of differentiation. The basophil method uses the resistance of basophils to acid lysis and differentiates them from the rest of the white blood cell population. The Advia 120 analyzer method of counting platelets is based on two-dimensional laser light scatter. The laser optics low angle and high angle scatter is used to determine the platelet count simultaneously with the red blood cells. Mean platelet volume (MPV) is a calculated parameter from platelet volume histogram. NLR, MLR, PLR, and MPV/PC were calculated as ratios of circulating neutrophil, monocyte, lymphocyte, and platelet counts, respectively. Normal ranges for these cell counts are as follows: leukocyte $4–10.8 \times 10^9$/L; neutrophil $1.9–8 \times 10^9$/L; lymphocyte $0.9–5.2 \times 10^9$/L; monocyte $0\text{-}1 \times 10^9$/L; and platelet $130.0–400.0 \times 10^9$/L (data from our laboratory).

2.3. Disease Severity and Outcome. To assess the severity of secondary sepsis and/or trauma, Acute Physiology and Chronic Health Evaluation (APACHE) II score was performed on admission in all critically ill patients, regardless of underlying condition. Injury Severity Score (ISS) (determined using Abbreviated Injury Scale (AIS)) was calculated and recorded in all trauma patients. Outcome measure was hospital mortality.

2.4. Statistical Analysis. In case of continuous data, variables were presented as mean value ± standard deviation (SD) or median followed by interquartile range. Some of the variables were presented as frequency of certain categories, while statistical significance of differences was tested with the chi square test. All variables were tested for normal distribution by Kolmogorov-Smirnov test. In accordance with the result of this test, the statistical significance of differences was tested using the *t*-test or Mann–Whitney *U* test (two groups' comparison). In case of multigroup comparison, ANOVA or Kruskal-Wallis test (post hoc Mann–Whitney test) was applied. The relationship between two variables was established using Spearman correlation analysis (rho value). The determination of cut-off values and sensitivity and specificity of the variables were analyzed using the ROC curve procedure (the Youden index was used in all cases). Calculations of odds ratios and their 95% confidence intervals were done to determine the strength of the association between risk factors and outcomes. For that purpose, the most promising independent variables (univariate analysis) as single risk factors were incorporated into binary logistic regression analyses (multivariate analysis). Differences between groups were considered significant at $p < 0.05$. Complete statistical analysis of the data was conducted with the statistical software package SPSS Statistics 18 (Chicago, Illinois, USA).

3. Results

3.1. Baseline Characteristics of the Study Population. During a 4-year study period, 392 critically ill and injured patients were enrolled (60.2% male; mean age 53.67 ± 18.26 years). Nonsurvivors were older (mean ± SD, years): 63.21 ± 15.02 versus survivors 45.66 ± 16.86 ($t = 10.89$; $p \leq 0.001$). According to diagnosis, they were divided into 4 subgroups: peritonitis ($n = 196$; 50.0%), pancreatitis ($n = 67$; 17.1%), trauma with secondary sepsis ($n = 83$; 21.2%), and trauma without sepsis ($n = 46$; 11.7%). According to blood culture, there were 65 (16.6%) patients with isolated Gram-positive pathogens, 15 (3.8%) with Gram-negative pathogens, 184 (46.9%) with polymicrobial blood culture, and 128 (32.7%) with negative blood culture. Overall hospital mortality was 45.7%; there were 179 nonsurvivors. Acute Physiology and Chronic Health Evaluation (APACHE) II score (mean ± SD) was 22.32 ± 3.7 in all critically ill patients, regardless of underlying condition. Injury Severity Score (ISS) (determined using Abbreviated Injury Scale (AIS)) was calculated and recorded in all trauma patients (mean ± SD): 26.36 ± 5.42. Baseline characteristics of all patients according to outcome are shown in Table 1.

Statistical analysis of all patients revealed that female mortality rate was significantly higher ($p \leq 0.001$), which was not surprising given that females dominantly suffered from peritonitis ($p \leq 0.001$). Peritonitis and pancreatitis were significantly associated with lethal outcome ($p \leq 0.001$). On the other hand, males dominantly were traumatized with the development of secondary sepsis ($p \leq 0.001$). All patients in the trauma subgroup had negative blood culture; those with trauma and secondary sepsis dominantly had polymicrobial blood culture ($p \leq 0.001$). Males dominantly had negative blood culture ($p \leq 0.001$) as expected because they comprised almost 70% of the trauma subgroup.

In Table 2, laboratory characteristics of all patients according to outcome are shown.

Lymphocyte, monocyte, and platelet counts were significantly higher, and MPV was significantly lower in survivors. NLR and MPV/PC levels were higher in nonsurvivors, difference reached high statistical significance. MLR and PLR values did not differ significantly between survivors and nonsurvivors.

In Table 3, baseline and laboratory characteristics of all patients according to blood cultures are shown.

Post hoc Mann–Whitney test revealed that both lymphocyte count and monocyte count were significantly higher in patients with Gram-positive blood culture compared to those with polymicrobial and negative blood cultures. Also, patients with Gram-positive blood culture had significantly lower MPV/PC ratio compared to patients with Gram-negative and polymicrobial blood cultures. When comparing polymicrobial and negative blood culture, we found significantly higher MPV and MPV/PC values as well as significantly lower platelet count in patients with polymicrobial blood culture. Patients with Gram-negative, polymicrobial, and negative blood cultures had significantly higher PLR and MLR values in comparison with those who had Gram-positive blood culture. When comparing polymicrobial and negative blood cultures, we found significantly lower PLR and MLR values in patients with polymicrobial blood culture. So, the highest levels of MLR and PLR had been found in patients with negative blood culture and the lowest in patients with Gram-positive blood culture. NLR values did not differ significantly between these four subgroups of patients according to blood culture.

In Table 4, baseline and laboratory characteristics of all patients according to underlying condition are shown.

Post hoc Mann–Whitney test revealed that platelet count was significantly higher in trauma patients compared to those with other underlying conditions (the lowest count was in the peritonitis subgroup). Patients with peritonitis and pancreatitis had significantly higher MPV values compared to trauma patients with and without secondary sepsis. Monocyte count, as well as MLR value, was significantly lower in patients with peritonitis compared to those with other underlying conditions. In the pancreatitis subgroup, NLR value was significantly higher compared to those with other underlying conditions. PLR and MPV/PC values did not differ significantly between these four subgroups of patients according to underlying condition.

3.2. Peritonitis Subgroup. There were 196 patients with peritonitis. Baseline and laboratory characteristics of the peritonitis subgroup are shown in Table 1 in Supplementary file. Statistical analysis of laboratory characteristics of the peritonitis subgroup according to outcome showed the same trend and significant differences as in all 392 patients. Clinical accuracy of baseline biomarkers in predicting lethal outcome in the peritonitis subgroup was investigated. Monocytes, lymphocytes, and platelets lower than cut-off values as well as MPV, MPV/PC, and NLR higher than cut-off values are

Table 1: Baseline characteristics of all patients according to outcome.

Characteristics	All patients	Survivors	Nonsurvivors
Number of patients (%)	392 (100%)	213 (54.3%)	179 (45.7%)
Males, number (%)	236 (60.2%)	149 (70%)	87 (48.6%)
Age (years)	53.67 ± 18.26	45.66 ± 16.86	63.21 ± 15.02
Peritonitis	196 (50.0%)	93 (47.4%)	103 (52.6%)
Pancreatitis	67 (17.1%)	28 (41.8%)	39 (58.2%)
Trauma + sepsis	83 (21.2%)	57 (68.7%)	26 (31.3%)
Trauma	46 (11.7%)	35 (76.1%)	11 (23.9%)
Gram-positive blood cultures, number (%)	65 (16.6%)	53 (24.9%)	12 (6.7%)
Gram-negative blood cultures, number (%)	15 (3.8%)	11 (5.2%)	4 (2.2%)
Polymicrobial blood cultures, number (%)	184 (46.9%)	53 (24.9%)	131 (73.2%)
Negative blood cultures, number (%)	128 (32.7%)	96 (45.1%)	32 (17.9%)

Table 2: Laboratory characteristics of all patients according to outcome.

Characteristics	All patients	Survivors	Nonsurvivors	p value
WBC count (10^9/L)	12.74 ± 5.59	12.91 ± 5.31	12.53 ± 5.91	$p = 0.312$
Neutrophil (10^9/L)	10.40 (7.32–13.42)	10.30 (7.30–13.75)	10.40 (7.40–13.05)	$p = 0.728$
Lymphocyte (10^9/L)	0.95 (0.61–1.31)	1.06 (0.77–1.45)	0.81 (0.55–1.20)	$p \leq 0.001^{**}$
Monocyte (10^9/L)	0.60 (0.35–0.92)	0.64 (0.40–1.03)	0.56 (0.29–0.80)	$p \leq 0.001^{**}$
Platelet (10^9/L)	241.63 ± 152.29	267.41 ± 156.04	210.94 ± 142.15	$p \leq 0.001^{**}$
MPV (fL)	9.05 ± 1.76	8.61 ± 1.27	9.61 ± 2.11	$p \leq 0.001^{**}$
MPV/PC ratio (fL 10^{-5} μL^{-1})	3.7 (2.5–6.2)	3.4 (2.3–5.3)	4.7 (3.0–8.0)	$p \leq 0.001^{**}$
NLR	11.00 (7.02–15.71)	9.91 (6.17–13.78)	12.26 (7.72–18.25)	$p = 0.001^{**}$
MLR	0.61 (0.38–1.02)	0.62 (0.37–1.02)	0.58 (0.39–1.01)	$p = 0.240$
PLR	226.53 (162.16–354.20)	228.38 (152.71–328.59)	225.11 (166.47–410.40)	$p = 0.848$

Data are shown as number (%), mean (standard deviation, SD), or median (interquartile range, IQR) as appropriate. Significant differences are marked by ** ($p < 0.01$).

moderate predictors of lethal outcome in patients with peritonitis (Table 2 in Supplementary file). Discriminative power of MLR and PLR regarding outcome in this subgroup of patients was not significant. Clinical accuracy of baseline biomarkers in predicting negative blood culture in this subgroup was also investigated. MPV and MPV/PC lower than cut-off values as well as platelets, MLR, and PLR higher than cut-off values are moderate predictors of negative blood culture in patients with peritonitis (Table 3 in Supplementary file). Discriminative power of other investigated biomarkers regarding negative blood culture in this subgroup of patients was not significant.

3.3. Pancreatitis Subgroup. There were 67 patients with pancreatitis. Baseline and laboratory characteristics of the pancreatitis subgroup are shown in Table 4 in Supplementary file. Lymphocyte count and platelet count were significantly higher in survivors, monocyte count showed the same trend, but it did not reach statistical significance. Similar trend, seen in all 392 patients as well as in the peritonitis subgroup, was evident in the pancreatitis subgroup (MPV, MPV/PC, and NLR levels were significantly higher in nonsurvivors; MLR and PLR values did not differ significantly between survivors and nonsurvivors). But, unlike the whole group and the peritonitis subgroup, in the pancreatitis subgroup of patients, there were significantly higher WBC and neutrophil counts in nonsurvivors. Clinical accuracy of baseline biomarkers in predicting lethal outcome in the pancreatitis subgroup was investigated. Lymphocytes and platelets lower than cut-off values as well as MPV, MPV/PC, and NLR higher than cut-off values are very good predictors of lethal outcome in patients with pancreatitis (Table 5 in Supplementary file). Discriminative power of monocyte, MLR, and PLR regarding outcome in this subgroup of patients was not significant. Clinical accuracy of baseline biomarkers in predicting negative blood culture in this subgroup was also investigated. Lymphocyte and platelet counts higher than cut-off values are moderate predictors whereas MPV, MPV/PC, and NLR lower than cut-off values are very good predictors of negative blood culture in patients with pancreatitis (Table 6 in Supplementary file). Discriminative power of other investigated biomarkers regarding negative blood culture in this group of patients was not significant.

3.4. Trauma Patients with Secondary Sepsis. There were 83 trauma patients who developed secondary sepsis. Baseline

Table 3: Baseline and laboratory characteristics of all patients according to blood cultures.

Characteristics	Gram-positive blood cultures	Gram-negative blood cultures	Polymicrobial blood cultures	Negative blood cultures	p value
Number of patients (%)	65 (16.6%)	15 (3.8%)	184 (46.9%)	128 (32.7%)	—
Males, number (%)	40 (61.5%)	11 (73.3%)	96 (52.2%)	89 (69.5%)	—
Age (years)	50.46 ± 23.81	59.33 ± 12.66	53.71 ± 17.55	54.59 ± 16.41	$p = 0.287$
Mortality (%)	18.5%	26.7%	71.2%	25.0%	—
WBC count (10^9/L)	13.95 ± 5.63	13.03 ± 4.27	12.20 ± 5.99	12.85 ± 5.02	$p = 0.113$
Neutrophil (10^9/L)	11.10 (8.05–14.25)	10.80 (9.09–15.40)	10.20 (6.43–13.30)	9.96 (7.41–13.40)	$p = 0.226$
Lymphocyte (10^9/L)	1.14 (0.84–1.60)	0.92 (0.60–1.07)	0.86 (0.58–1.26)	0.97 (0.72–1.41)	$p \leq 0.001^{**}$
Monocyte (10^9/L)	0.63 (0.43–0.95)	0.48 (0.28–1.15)	0.56 (0.29–0.81)	0.67 (0.38–1.04)	$p = 0.016^{*}$
Platelet (10^9/L)	229.02 ± 110.53	280.26 ± 167.22	215.99 ± 134.50	280.35 ± 182.70	$p = 0.019^{*}$
MPV (fL)	8.77 ± 1.49	9.11 ± 1.37	9.40 ± 1.99	8.69 ± 1.50	$p = 0.010^{*}$
MPV/PC ratio (fL $10^{-5}\,\mu L^{-1}$)	3.5 (2.4–5.7)	5.3 (2.3–5.4)	4.4 (2.8–7.7)	3.5 (2.2–5.7)	$p = 0.012^{*}$
NLR	9.71 (5.84–12.52)	11.44 (7.37–17.41)	11.13 (7.18–17.08)	11.35 (6.71–15.39)	$p = 0.243$
MLR	0.44 (0.33–0.93)	0.54 (0.38–0.80)	0.57 (0.35–1.03)	0.67 (0.42–1.13)	$p = 0.040^{*}$
PLR	197.70 (139.53–274.90)	228.38 (191.26–376.71)	220.95 (161.51–376.52)	264.00 (168.08–378.01)	$p = 0.010^{*}$

Data are shown as number (%), mean (standard deviation, SD), or median (interquartile range, IQR) as appropriate. Significant differences are marked by * ($p < 0.05$) or ** ($p < 0.01$).

Table 4: Baseline and laboratory characteristics of all patients according to underlying condition.

Characteristics	Peritonitis subgroup	Pancreatitis subgroup	Trauma + sepsis subgroup	Trauma subgroup	p value
Number of patients (%)	196 (50.0%)	67 (17.1%)	83 (21.2%)	46 (11.7%)	—
Males, number (%)	79 (40.3%)	48 (71.6%)	77 (92.8%)	32 (69.6%)	—
Age (years)	59.58 ± 17.78	54.15 ± 13.04	39.60 ± 14.86	53.20 ± 18.93	$p \leq 0.001^{**}$
Mortality (%)	52.6%	58.2%	31.3%	23.9%	—
WBC count (10^9/L)	12.83 ± 5.74	12.30 ± 4.06	12.55 ± 6.29	13.32 ± 5.64	$p = 0.794$
Neutrophil (10^9/L)	10.55 (8.08–13.72)	10.60 (7.55–12.80)	10.00 (5.59–13.70)	10.04 (7.35–13.92)	$p = 0.621$
Lymphocyte (10^9/L)	0.95 (0.58–1.29)	0.92 (0.69–1.28)	1.02 (0.64–1.48)	0.95 (0.58–1.28)	$p = 0.431$
Monocyte (10^9/L)	0.54 (0.27–0.85)	0.67 (0.50–0.89)	0.61 (0.41–0.89)	0.67 (0.39–1.14)	$p = 0.005^{**}$
Platelet (10^9/L)	228.56 ± 136.88	233.90 ± 123.08	245.64 ± 131.20	301.31 ± 249.50	$p = 0.032^{*}$
MPV (fL)	9.15 ± 1.68	9.67 ± 2.51	8.44 ± 0.96	8.90 ± 1.62	$p \leq 0.001^{**}$
MPV/PC ratio (fL $10^{-5}\,\mu L^{-1}$)	3.6 (2.7–6.2)	4.4 (2.2–6.6)	3.6 (2.4–5.7)	4.2 (2.0–6.5)	$p = 0.794$
NLR	11.20 (7.87–17.25)	11.35 (6.48–15.74)	9.10 (5.08–15.19)	11.13 (7.64–14.59)	$p = 0.035^{*}$
MLR	0.51 (0.34–0.94)	0.67 (0.46–1.16)	0.59 (0.33–0.94)	0.71 (0.45–1.21)	$p = 0.002^{**}$
PLR	227.90 (167.65–362.89)	223.28 (140.65–377.88)	210.41 (147.89–337.60)	243.24 (148.22–380.69)	$p = 0.652$

Data are shown as number (%), mean (standard deviation, SD), or median (interquartile range, IQR) as appropriate. Significant differences are marked by * ($p < 0.05$) or ** ($p < 0.01$).

and laboratory characteristics of the trauma + sepsis subgroup are shown in Table 7 in Supplementary file. Unlike patients with peritonitis and pancreatitis, there was no significant difference of investigated laboratory parameters between survivors and nonsurvivors in trauma patients who developed secondary sepsis. Therefore, in this subgroup, none of the investigated biomarkers had significant discriminative power regarding outcome. Clinical accuracy of baseline biomarkers in predicting polymicrobial blood culture in trauma patients with secondary sepsis was investigated. Lymphocyte count lower than cut-off value and NLR higher than cut-off value are moderate predictors of polymicrobial

blood culture in trauma patients with secondary sepsis (Table 8 in Supplementary file). Discriminative power of other investigated biomarkers regarding polymicrobial blood culture in this subgroup of patients was not significant.

3.5. Trauma Subgroup. There were 46 trauma patients. Baseline and laboratory characteristics of the trauma subgroup are shown in Table 9 in Supplementary file. Unlike all other subgroups of patients, in trauma patients, PLR value was significantly higher in nonsurvivors. Also, in this subgroup, all other laboratory parameters showed no significant difference between survivors and nonsurvivors. Clinical accuracy of baseline biomarker PLR in predicting lethal outcome in the trauma subgroup was investigated. PLR higher than cut-off value is a very good predictor of lethal outcome in trauma patients (Table 10 in Supplementary file). Discriminative power of all other biomarkers regarding outcome in this subgroup of patients was not significant.

3.6. Combination of MPV/PC, NLR, MLR, and PLR in a Composite Bioscore. In order to determine whether combination of these four ratios into a one composite bioscore is going to improve their prognostic performance regarding lethal outcome in all subgroups of patients, individual values were scored as 1 or 0 based on their relation (below or above) to previously established ROC curve cut-off levels. Bioscore ranges from 0 to 4 points.

In the peritonitis subgroup, AUC/ROC for composite bioscore was 0.718 (95% confidence interval 0.644–0.792; $p \leq 0.001$). This is shown in Figure 1B in Supplementary file. Composite bioscore of 1 point had a sensitivity of 63.5% and a specificity of 68.0% (Youden index = 0.31). Bioscore higher than this cut-off value is a good predictor of lethal outcome. In Figure 1B in Supplementary file, percentage of nonsurvivors for each bioscore point value is shown.

In the pancreatitis subgroup, AUC/ROC for composite bioscore was 0.874 (95% confidence interval 0.791–0.956; $p \leq 0.001$). This is shown in Figure 1(a). Composite bioscore of 2 points had a sensitivity of 72.5% and a specificity of 79.5% (Youden index = 0.51). Bioscore higher than this cut-off value is a very good predictor of lethal outcome. In Figure 1(b), percentage of nonsurvivors for each bioscore point value is shown.

In the trauma with secondary sepsis subgroup, AUC/ROC for composite bioscore was 0.782 (95% confidence interval 0.677–0.888; $p \leq 0.001$). This is shown in Figure 2A in Supplementary file. Composite bioscore of 2 points had a sensitivity of 71.1% and a specificity of 68.2% (Youden index = 0.39). Bioscore higher than this cut-off value is a good predictor of lethal outcome. In Figure 2B in Supplementary file, percentage of nonsurvivors for each bioscore point value is shown.

In the trauma subgroup, AUC/ROC for composite bioscore was 0.823 (95% confidence interval 0.673–0.974; $p = 0.001$). This is shown in Figure 3B in Supplementary file. Composite bioscore of 3 points had a sensitivity of 50.0% and a specificity of 94.3% (Youden index = 0.44). Bioscore higher than this cut-off value is a good predictor of lethal outcome. In Figure 3B in Supplementary file, percentage of nonsurvivors for each bioscore point value is shown.

3.7. Correlation between MPV/PC, NLR, MLR, and PLR. A Spearman rho test of correlation between MPV/PC, NLR, MLR, and PLR was performed. In general, regarding all patients, there were significantly positive correlations between investigated biomarkers, regardless of outcome (Table 11 in Supplementary file). In both survivors and nonsurvivors, there was a significantly positive correlation between PLR and NLR or MLR; there was a significantly negative correlation between PLR and MPV/PC (Figure 2).

Also, there was a significantly positive correlation between MLR and NLR. The correlation between MPV/PC and NLR or MLR was not significant.

In the peritonitis subgroup, there was exactly the same trend of correlations between same biomarkers, with same levels of significance.

In the pancreatitis subgroup, the same trend of correlations with same biomarkers and levels of significance continued with one addition: there was a significantly negative correlation between MLR and MPV/PC in nonsurvivors (rho = −0.418; $p = 0.009$).

In trauma with secondary sepsis nonsurvivors, the same trend of correlations with same biomarkers and levels of significance continued. But, in survivors from this subgroup, trend was slightly different: correlation between PLR and MLR was not significant.

In the trauma subgroup, there was a different trend of correlations in both survivors and nonsurvivors (Table 12, Figure 4 in Supplementary file). In both survivors and nonsurvivors, there was a significantly negative correlation between PLR and MPV/PC; also, there was a significantly negative correlation between PLR and MLR in nonsurvivors and a significantly positive correlation between same two biomarkers in survivors. Also, there was a significantly positive correlation between MLR and MPV/PC in nonsurvivors as well as between MLR and NLR in survivors. Other correlations were not significant.

In survivors, regardless of underlying condition, there was exactly the same trend of correlations between same biomarkers, with same levels of significance in patients with Gram-positive and negative blood cultures (Table 13 in Supplementary file).

In survivors with Gram-positive and negative blood cultures, there was a significantly positive correlation between PLR and NLR or MLR; there was a significantly negative correlation between PLR and MPV/PC. Also, there was a significantly positive correlation between MLR and NLR. The correlation between MPV/PC and NLR or MLR was not significant.

In survivors with polymicrobial blood cultures, there was a significantly negative correlation between PLR and MPV/PC (rho = −0.566; $p \leq 0.001$) as well as a significantly positive correlation between PLR and NLR (rho = 0.310; $p = 0.024$). Other correlations were not significant.

In survivors with Gram-negative blood cultures, the only significant correlation was positive one between NLR and MPV/PC (rho = 0.618; $p = 0.030$).

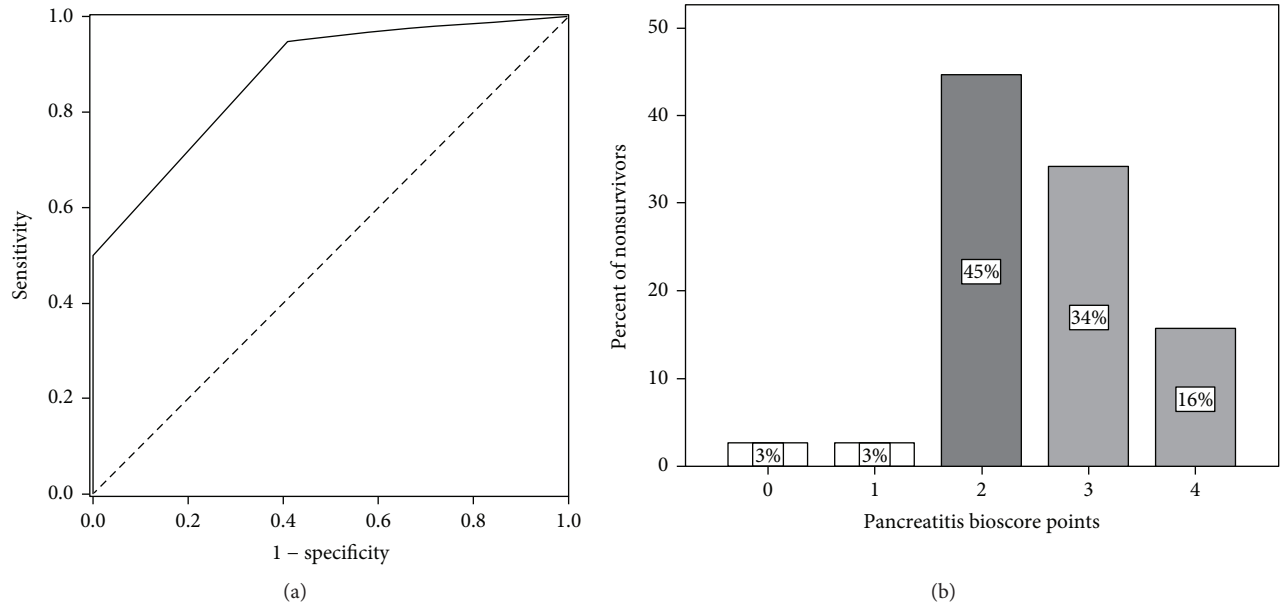

Figure 1: (a) Receiver operating characteristic (ROC) curve for composite bioscore in the pancreatitis subgroup and the lethal outcome (AUC = 0.874). (b) Percentage of nonsurvivors according to each bioscore point value in the pancreatitis subgroup (darkest shade: cut-off bioscore point value).

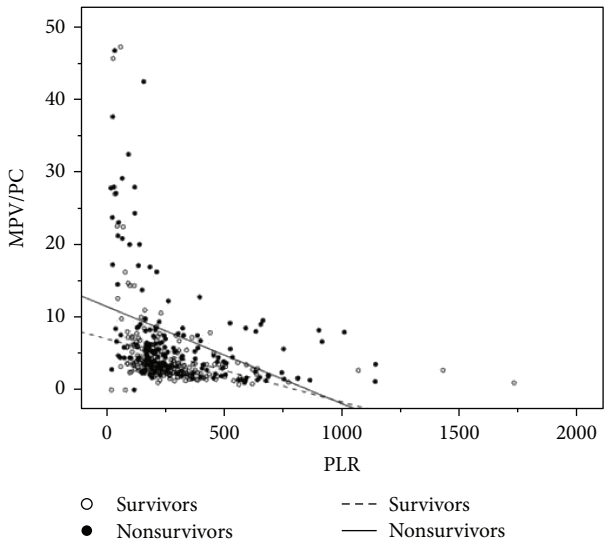

Figure 2: Scattergram of MPV/PC versus PLR for all patients (survivors and nonsurvivors).

There were no significant correlations between investigated biomarkers in nonsurvivors with Gram-negative blood cultures.

In nonsurvivors with Gram-positive and polymicrobial blood cultures, there was a similar trend in correlations between investigated biomarkers (Table 14 in Supplementary file).

In nonsurvivors with negative blood cultures, there were only two significant correlations between MPV/PC on one hand and NLR (positive correlation; rho = 0.590; $p = 0.001$) and PLR (negative correlation; rho = −0.633; $p \leq 0.001$) on the other hand.

3.8. Independent Prognostic Significance of Cells and Ratios in Predicting Lethal Outcome. Univariate logistic regression analyses were performed in order to determine whether associations of each individual variable with lethal outcome exist. Standardized regression coefficient (β) and the odds ratio (OR) with 95% CI were calculated for each variable. Forward stepwise multivariate logistic regression model was performed in order to determine the independent predictors of lethal outcome, without the effect of possible confounders. In Table 5, univariate odds ratios of variables for predicting lethal outcome in all patients are shown.

Independent predictors of lethal outcome by multivariate logistic regression analysis in all patients are shown in Table 6.

Among categorical variables, the most important independent predictor of lethal outcome is polymicrobial blood culture, followed by female gender and pancreatitis as underlying condition, respectively. As far as continuous variables are concerned, the most important independent predictor of lethal outcome is older age, then comes higher NLR and lower platelet count, respectively. Multivariate logistic regression analysis demonstrated that polymicrobial blood culture remained statistically highly significant independent predictor of lethal outcome when compared to both Gram-positive (standard β value 3.950; OR 51.922, 95% CI 13.777–195.681; $p \leq 0.001$) and Gram-negative (standard β value 3.117; OR 22.584, 95% CI 4.465–114.231; $p \leq 0.001$) blood cultures.

Univariate and multivariate logistic regression analyses were performed in the same fashion in the peritonitis subgroup. Here again, the peritonitis subgroup showed very similar trend compared to the whole cohort of critically ill and injured patients (Tables 15 and 16 in Supplementary file).

Table 5: Univariate odds ratios of variables for predicting lethal outcome in all patients.

Variables	Standard β value	OR	95% confidence interval		p value
			Lower bound	Upper bound	
Female gender	0.901	2.462	1.627	3.726	0.001**
Age	0.064	1.066	1.050	1.081	0.001**
Underlying condition (peritonitis)	1.260	3.524	1.693	7.336	0.001**
Underlying condition (pancreatitis)	1.489	4.432	1.926	10.199	0.001**
Underlying condition (trauma with secondary sepsis)	0.372	1.451	0.639	3.299	0.374
Gram-positive blood culture	−0.387	0.679	0.323	1.429	0.308
Gram-negative blood culture	0.087	1.091	0.325	3.667	0.888
Polymicrobial blood culture	2.004	7.415	4.445	12.370	0.001**
WBC	−0.012	0.988	0.953	1.024	0.508
Neutrophil	−0.001	0.999	0.961	1.038	0.952
Lymphocyte	−0.223	0.800	0.592	1.083	0.149
Monocyte	−0.935	0.393	0.243	0.635	0.001**
Platelet	−0.003	0.997	0.996	0.999	0.001**
MPV	0.361	1.435	1.247	1.651	0.001**
MPV/PC	0.069	1.072	1.030	1.114	0.001**
NLR	0.036	1.037	1.014	1.060	0.001**
MLR	−0.171	0.842	0.590	1.202	0.344
PLR	0.001	1.001	1.000	1.001	0.286

Significant differences are marked by ** ($p < 0.01$).

Table 6: Independent predictors of lethal outcome by multivariate logistic regression analysis in all patients.

Variables	Standard β value	OR	95% confidence interval		p value
			Lower bound	Upper bound	
Female gender	1348	3851	1712	8664	0.001**
Age	0.115	1.122	1.089	1.155	0.001**
Underlying condition (pancreatitis)	1.184	3.266	1.120	11.234	0.040*
Polymicrobial blood culture[#]	3.739	42.074	14.174	124.893	0.001**
Platelet	−0.004	1.004	1.001	1.007	0.017*
NLR	0.049	1.051	1.013	1.089	0.007**

[#]Polymicrobial blood culture compared to negative blood culture. Significant differences are marked by * ($p < 0.05$) or ** ($p < 0.01$).

Among categorical variables, the most important independent predictor of lethal outcome is polymicrobial blood culture, followed by female gender. As far as continuous variables are concerned, the most important independent predictor of lethal outcome is older age, then comes higher NLR value. Multivariate logistic regression analysis demonstrated that polymicrobial blood culture remained statistically highly significant independent predictor of lethal outcome when compared to both Gram-positive (standard β value 3.539; OR 34.446, 95% CI 7.422–159.874; $p \leq 0.001$) and Gram-negative (standard β value 2.710; OR 15.034, 95% CI 2.633–85.828; $p = 0.002$) blood cultures.

Slightly different trend was demonstrated in the pancreatitis subgroup regarding univariate and multivariate logistic regression analyses of lethal outcome predictors (Tables 17 and 18 in Supplementary file). As far as continuous variables are concerned, the most important independent predictor of lethal outcome is higher MVP value, then older age and lower lymphocyte count, respectively.

In patients with and without secondary trauma, univariate logistic regression analyses demonstrated that only older age is the significant independent predictor of lethal outcome (trauma with secondary sepsis subgroup: standard β value 0.075; OR 1.078, 95% CI 1.036–1.121; $p \leq 0.001$; trauma subgroup: standard β value 0.222; OR 1.249, 95% CI 1.063–1.467; $p = 0.007$).

4. Discussion

Our prospective observational study focused on prognostic value of four different cell ratios, NLR, MLR, PLR, and MPV/PC, primarily regarding lethal outcome and secondarily

regarding nature of blood cultures in a heterogeneous cohort of critically ill patients with secondary sepsis and/or trauma. We chose these parameters because of their availability in routine clinical practice in a real-life setting. We analyzed our critically ill patient population cohort as a whole, as well as divided it into four subgroups according to underlying condition separately, due to heterogeneity.

The key cell types of the innate immune system as well as the first cellular line of defense against infection are neutrophils. Lymphocytes are involved in adaptive immune response. Immune response to various insults frequently has distinctive feature: increase in neutrophil count and decrease in lymphocyte count. When infection persists, large amount of neutrophils is produced and they might not become apoptotic. Apoptosis of neutrophils in sepsis is beneficial, in contrast to lymphocytes. In this study, circulating cell counts were investigated. Next, logical step would be adding phenotypic markers into analysis. But, swift implementation of these markers is hampered by costs and accessibility.

In a study regarding NLR as predictor of lethal outcome in sepsis, Liu and coworkers [3] reported results similar to ours. NLR was significantly higher in nonsurvivors, with slightly higher AUC than in our study, and in our investigation, NLR was an independent predictor of lethal outcome. In that study, other ratios were not explored, and unlike our results, they reported no significant difference in platelet count between survivors and nonsurvivors. Also, their outcome measure was 28-day mortality and ours was hospital mortality, which is, in our opinion, a better choice. Either way, choice of outcome measure influences results. Association between NLR and mortality in critically ill was investigated by Salciccioli and coworkers in an observational cohort study [8]. They found a statistically significantly higher baseline NLR values in patients with sepsis compared to those without sepsis contrary to our results. Also, opposite to our results, they found the strongest relationship between NLR and mortality in patients without sepsis. In our study, it is the trauma subgroup in which there was no significant difference in NLR values between survivors and nonsurvivors. These authors found an association between NLR and outcome in whole cohort of critically ill patients like we did. In accordance with our results regarding NLR in pancreatitis subgroup are findings reported by Azab and coauthors [9]. They also found that NLR was a good predictor of adverse outcome of acute pancreatitis in ICU setting. In a different population of patients with the first episode of community-acquired or healthcare-related bacteremia during hospital admission, Terradas and coauthors found that persistence of an $NLR > 7$ was an independent marker of mortality [10]. Trend was the same in our study, only NLR values with the best sensitivity and specificity as predictors of lethal outcome were higher: in the peritonitis subgroup 13.25 and in the pancreatitis subgroup 10.44, which is not surprising given that our patient population was critically ill in ICU setting. Interesting study regarding reversal of NLR in early (in the first week) versus late (within or beyond the first month) death from septic shock was conducted by Riché and coauthors [11]. In the whole cohort of 130 patients, authors found that neutrophil count at admission was similar between survivors and nonsurvivors. Same was true in our study except for the pancreatitis subgroup. They also found that lymphocyte count was higher and NLR reduced in nonsurvivors. These findings are in contrast to those in our study regarding nonsurvivors: lymphocyte count was lower in the whole cohort as well as in all subgroups of our patients and NLR was significantly higher in the whole cohort, the peritonitis subgroup, and the pancreatitis subgroup, while in trauma patients with and without sepsis NLR was lower in nonsurvivors, but it did not reach statistical significance. In the literature available to us, studies regarding cell counts and ratios in general—and NLR specifically in trauma patients—are sparse at best and almost always focused on surgical patients [12]. In a large cohort study on 1356 adult trauma patients (median ISS of 13, comparable to our trauma subgroups), admitted to the surgical ICU of a level 1 trauma center, predictive capacity of NLR on mortality was assessed [13]. Authors performed ROC curve analyses at ICU days 2 and 5, and they found optimal NLR cut-off values of 10.45 and 7.91, respectively. They calculated cut-off values by maximizing the Youden index as we did. Also, in their study, NLR greater than or equal to these cut-off values was a marker for increased hospital mortality. These findings are in contrast to those in our study regarding trauma nonsurvivors; NLR was insignificantly lower in trauma with and without sepsis nonsurvivors, with no significant discriminative power regarding outcome. NLR has good prognostic accuracy regarding outcome, generally higher than traditional infection markers, not only in sepsis but also in a variety of infective states [14].

Several studies investigated diagnostic value of NLR for bacteremia. In emergency department setting, de Jager and coauthors found significant differences between patients with positive and negative blood cultures in lymphocyte count and NLR [15]. Patients with positive cultures had lower lymphocyte count (0.8 versus 1.2×10^9/L) and higher NLR (20.9 versus 13.2); also, AUC/ROC of 0.73 for both biomarkers was high. These results were in accordance with our results. We investigated clinical accuracy of cell counts and ratios in predicting negative blood cultures in the peritonitis subgroup and the pancreatitis subgroup. Higher lymphocyte count (AUC 0.68) and lower NLR (AUC 0.72) were good predictors of negative blood culture in the pancreatitis group; interestingly, their discriminative power in the peritonitis group was not significant. Similar results regarding evaluation of NLR as a diagnostic biomarker for positive versus negative blood cultures were reported by Zhang with coauthors [16]. NLR values were significantly higher in patients with positive blood cultures, diagnostic performance was good (AUC 0.71) as in our pancreatitis subgroup. In these two studies [15, 16], underlying condition leading to sepsis was not reported, in one of them [15] comorbidities were. Our cut-off value for NLR in the pancreatitis subgroup was 13.00;

this is in accordance with the proposed appropriate cut-off value of NLR for sepsis (13–15) [17].

Monocytes are an essential component of the innate immune response that acts as a link to the adaptive immune system via antigen presentation to lymphocytes. Literature regarding MLR in infection is scarce. One study investigated NLR and MLR in discriminating between different patient groups hospitalized for fever due to infection and those without infection [4]. They concluded that both NLR and MLR may be useful in the diagnosis of bacterial infection, AUCs 0.708 and 0.688, respectively. Also, authors reported that the highest MLR had been found in patients with confirmed bacterial infection and lower MLR was found in patients with clinically diagnosed infection which was not supported by microbiology. This is in contrast to MLR values in our study: MLR was a moderate predictor of negative blood culture in the peritonitis subgroup with AUC of 0.586 ($p = 0.046$); patients with negative blood culture had significantly higher MLR values. Variability of MLR values was demonstrated in study regarding this cell ratio in patients with active tuberculosis [18]. Authors found that patients with active tuberculosis had a higher or lower MLR compared to controls and that MLR in extreme percentiles were significant predictors of active tuberculosis (<9% or >25%).

MLR was assessed as a predictor of survival in patients with various malignant diseases [19]. In one study, authors concluded that this ratio predicts patient survival and aggressiveness of endometrial cancer [20]. Given that there is an association between inflammation and cancer and that chronic inflammation is important in the malignant transformation, promotion, and metastasis of cancer, we investigated MLR as a predictor of lethal outcome. Differences between MLR values in survivors and nonsurvivors were not significant in neither of our subgroup of patients or in the whole cohort. Univariate and multivariate logistic regression analyses confirmed that MLR has no independent prognostic significance regarding outcome.

Systemic inflammation is an integral part of disease progression in critical illness and is commonly associated with sepsis, leading to an increased risk of mortality. Prognostic value of PLR in critically ill patients with acute kidney injury was assessed by Zheng and coauthors [5]. They observed a U-shaped relationship between PLR and both 90-day and 30-day mortality, with the lowest risk being at values ranging from 90 to 311, so both low and high PLRs were associated with increased mortality. Authors concluded that PLR appears to be a novel, independent prognostic marker of outcome. This is in partial contrast to PLR values in our study except for our trauma subgroup. In traumatized patients, nonsurvivors had significantly higher PLR values. Interestingly, in this subgroup of our patient population, all other laboratory parameters showed no significant difference between survivors and nonsurvivors. PLR higher than cut-off value was a very good predictor of lethal outcome (AUC 0.719; $p = 0.030$). Contrary to our PLR results in the trauma subgroup of critically ill patients, Emektar and coauthors reported that in somewhat different patient population of older patients with hip fractures, PLR values were higher in survivors, but with rather low discriminative power (AUC 0.56) [21]. In our study, PLR values were highest in patients with negative blood culture in the whole cohort of critically ill patients, and it was statistically highly significant. PLR also had moderate discriminative power in predicting negative blood culture in the peritonitis subgroup (AUC 0.613; $p = 0.020$). In accordance with our results are findings from Bekdas and Ozturk, who, in different clinical setting but also related to infection, assessed diagnostic accuracy of PLR regarding acute complicated appendicitis (defined as a the presence of phlegmon, abscess, or perforation) in pediatric population [22]. In their study, in diagnosis of complicated acute appendicitis, PLR had a sensitivity of 62.5% and a specificity of 61.8%. So, PLR, as a novel biomarker, has been investigated recently in various clinical settings [23, 24].

Sepsis is associated with hemostatic system dysfunction, and platelets play important role in both hemostasis and immunoinflammatory response to various insults. Platelet count is inversely associated with MPV. Secretory granules in platelets are related to cell reactivity in hemostatic regulation. Platelets with higher MPV may have more granules and larger surface area, and this is associated with their activation. Also, platelets express Toll-like receptors (TLRs) so they recognize various molecular patterns in microorganisms. This leads to platelet activation. MPV/PC has recently been investigated as a promising predictor of mortality in critically ill sepsis patients. One such study included 120 sepsis patients, and clinical outcome was 28-day mortality [6]. Authors reported that higher MPV/PC ratio, specifically >3.71, on admission was a significant risk factor for 28-day mortality (AUC 0.81; $p = 0.001$). In their study, MPV or PC alone did not predict mortality; however, MPV/PC did and was an independent predictor of 28-day mortality. In comparison, our data showed that nonsurvivors had statistically significantly lower PC and higher MPV and MPV/PC. Clinical accuracy of MPV/PC was similar, this ratio was a good predictor of lethal outcome (similar cut-off value of 3.80, AUC 0.68; $p \leq 0.001$). But, unlike that study, our data showed that both PC and MPV were good predictors of outcome (AUCs 0.68 and 0.63, resp.; $p < 0.010$ in both) in the peritonitis subgroup. Same was true for the pancreatitis group with even better clinical accuracy (PC: AUC 0.69, $p = 0.006$; MPV: AUC 0.79, $p \leq 0.001$; MPV/PC: AUC 0.71, $p = 0.003$). This was confirmed by univariate odds ratios for predicting lethal outcome in the whole cohort of critically ill and injured patients. All three variables had high statistical significance. PC was an independent predictor of lethal outcome by multivariate logistic regression analysis in the whole cohort as well as MPV in the pancreatitis subgroup. So, in our study, the MPV/PC ratio was not superior to MPV or PC alone. In accordance with our results are those reported by Gao and coauthors [25]. In their investigation, a total of 124 septic shock patients were enrolled and they showed that, among platelet indices, MPV had the highest AUC of 0.81 with similar cut-off value as in our study.

Several studies investigated prognostic value of MPV regarding outcome in critically ill sepsis patients with contradictory results. In accordance with our data are results from investigation conducted by Kim and coauthors, who reported that change in MPV between hospital admission and 72 hours was an independent predictor of 28-day mortality in 345 critically ill patients. They also found that baseline MPV is a good predictor of lethal outcome with AUC of 0.65. Conclusion was that an increase in MPV during the first 72 hours of hospitalization is an independent risk factor for adverse clinical outcome in patients with severe sepsis and/or septic shock [26]. In contrast, while Zampieri and coauthors reported that an increase in MPV after admission to an ICU is independently associated with higher hospital mortality in 84 critically ill patients [27], in their study, there was no statistically significant difference between survivors and nonsurvivors regarding baseline MPV at admission. Opposite to our results, Sadaka and coauthors reported that there was no relation between MPV on day 1 of septic shock and mortality with AUC of 0.5 in large retrospective analysis. There was no statistically significant difference between survivors and nonsurvivors regarding MPV on day 1 [28]. Effect of severe sepsis on platelet count and their indices was the focus of investigation reported by Guclu and coauthors. They enrolled 145 patients with sepsis and 143 patients as control group. PC and MPV were compared between sepsis survivors and nonsurvivors. PC was only marginally different; MPV was not statistically different. But, regarding power of discrimination between septic patients and controls, MPV had very good clinical accuracy in predicting sepsis with AUC of 0.75. It was confirmed by multivariate logistic regression model in which MPV was an independent predictor of sepsis, so it can be used as diagnostic tool [29]. Statistically different MPV and MPV/PC values were found between sepsis patients and controls in another study where authors did not perform univariate and multivariate logistic regression analysis [30].

The immunoinflammatory response in critically ill septic patients is very complex with fundamental differences in the host immune response to Gram-positive bacterial pathogens compared with Gram-negative microorganisms [31–40]. In our previous study [41], we also demonstrated significant difference in cytokine profile in severe Gram-positive and Gram-negative abdominal sepsis. Therefore, our secondary endpoint in this investigation was to assess possible differences of cell ratios regarding different blood cultures in patients with documented bacteremia given that these ratios are comprised of immunocompetent cells exhibiting different behavior in different bacterial settings. When analyzing baseline and laboratory characteristics of all patients according to blood cultures, our data showed that both lymphocyte and monocyte counts were significantly higher in patients with Gram-positive blood culture compared to those with polymicrobial and negative blood cultures. Also, patients with Gram-positive blood culture had significantly lower MPV/PC ratio compared to patients with Gram-negative and polymicrobial blood cultures. When comparing polymicrobial and negative blood cultures, we found significantly higher MPV and MPV/PC values as well as significantly lower platelet count in patients with polymicrobial blood culture. Patients with Gram-negative, polymicrobial, and negative blood cultures had significantly higher PLR and MLR values in comparison with those who had Gram-positive blood culture. When comparing polymicrobial and negative blood culture, we found significantly lower PLR and MLR values in patients with polymicrobial blood culture. So, the highest levels of MLR and PLR had been found in patients with negative blood culture and lowest in patients with Gram-positive blood culture. NLR values did not differ significantly between these four subgroups of patients according to blood culture. As mentioned before, lymphocyte and platelet counts, MPV, MPV/PC, MLR, NLR, and PLR were good predictors of negative blood cultures either in the peritonitis subgroup or the pancreatitis subgroup; most of the investigated biomarkers had good discriminative power regarding negative blood cultures in both subgroups. Multivariate logistic regression analysis revealed that polymicrobial blood culture (compared to negative blood culture) is an independent predictor of lethal outcome in our cohort of critically ill and injured patients.

Surprisingly, in the literature available to us, we found only one study regarding infection-specific status of MPV in adults with sepsis [42]. Authors reported that MPV measurements from the first and second days were significantly lower in patients with Gram-positive bacteria than in patients infected with other microorganisms. This is in accordance with our findings that MPV was significantly lower in patients with Gram-positive blood culture. Apart from few studies evaluating specific platelet and MPV responses to different types of microorganisms in septic mostly very low birth weight neonates, we could not find any other study regarding MPV. To the best of our knowledge, our study is the first to investigate MPV/PC, NLR, MLR, and PLR in adult population of critically ill septic patients and patients with severe trauma regarding nature of bacteremia. We think that it might be clinically useful to help initiate adequate antibiotic therapy. According to guidelines, broad spectrum antibiotics are administered as soon as possible. The lack of causative pathogen identification in more than 40% of cases will make it more difficult to select appropriate antibiotics and may have deleterious effects on the survival of critically ill septic patients.

Our composite bioscore, in which four investigated ratios were combined, in each subgroup, significantly improved their prognostic performance regarding lethal outcome, all AUCs were around 0.80. Combination of biomarkers, in that regard, was investigated by other authors with similar results [43–46].

There is important question regarding specific timeline of investigated biomarker measurements during the first 24 hours and their predictive value regarding outcome in critically ill patients. In one study, several biomarkers like MPV, platelet count (PC), PDW (platelet distribution width)/PC, and MPV/PC were evaluated as predictors of

lethal outcome [6]. Authors demonstrated that both baseline values and values at 24 hours of all four biomarkers were significantly different between survivors and nonsurvivors, with baseline MPV and PC being exception. At 48 and 72 hours, almost all of the investigated biomarkers lost their prognostic ability. Authors demonstrated that MPV/PC ratio in the early phase of severe sepsis (baseline and at 24 hours) was an independent predictor of 28-day mortality. In another study, baseline values of both PC and MPV were not significantly different between survivors and nonsurvivors. At 24 and 48 hours, only PC was significantly different, MPV was not; at 72 hours, both were significantly different between survivors and nonsurvivors. Authors also calculated prognostic value of difference in both biomarkers, ΔMPV_{24h} and ΔPC_{24h}, and demonstrated that both have good prognostic value regarding outcome [27]. Since repetitive measurements during the first 24 hours have not been reported in majority of studies, this question remains open. When repetitive measurements do exist, difference is always a useful parameter and might have similar or higher prognostic value than a single measurement.

Temporal variation of investigated biomarkers is another important issue. We found two studies regarding NLR with repetitive measurements. In the first one, authors demonstrated that NLR both at admission and at day 5 was statistically different between septic shock survivors and nonsurvivors. They also calculated cell counts and NLR variations from day 1 to day 5; all showed statistically significant difference between two groups [11]. The other study was focused on the impact of NLR on mortality in critically ill trauma patients. Authors demonstrated that NLRs over 10.45 and 7.91 are independent predictors of in-hospital mortality at days 2 and 5, respectively [13]. Our group, being well aware of multiple measurement importance, recently published a study regarding NLR in a different setting, pediatric acute appendicitis (AA). NLR was measured in three different time points with regard to surgery: preoperative, on day 1, and day 3 postoperatively. We demonstrated that NLR provides good monitoring of progression of AA in children and that its cutoff values may help in distinguishing the phases of AA; therefore, it could be used in diagnosis of AA in pediatric population [47]. In the literature available to us, we did not find any studies regarding MLR and PLR with repetitive measurements. Several studies focused on repetitive measurements of PC and MPV and their prognostic value regarding outcome in patients with sepsis and/or septic shock. Kim and coauthors investigated whether the change in MPV between hospital admission and 72 hours ($\Delta MPV_{72h\text{-}adm}$) predicts 28-day mortality in severe sepsis and/or septic shock [26]. Authors demonstrated that the rate of MPV increase was significantly higher in nonsurvivors and that, in multivariate analysis, $\Delta MPV_{72h\text{-}adm}$ was an independent predictor of 28-day mortality. It should be noted that both baseline MPV and $\Delta MPV_{72h\text{-}adm}$ had good clinical accuracy in predicting lethal outcome, with AUCs of 0.65 and 0.69, respectively. In another study, authors investigated the impact of various platelet indices as prognostic markers of septic shock. Among them were PC and MPV, all with repetitive measurements, from baseline, first five consecutive days, penultimate, and last day of hospital stay, so there were 8 time points altogether [25]. Clinical accuracy of PC and MPV in predicting lethal outcome was very different but consistent within all time points for each biomarker. AUCs for PC were low in all time points, all below 0.5, whereas AUCs for MPV were high in all time points, ranging from 0.66 to 0.88.

Next step in our research is to analyze different subsets of lymphocytes and to explore phenotypic markers. This is the focus of our forthcoming study.

Our present study has several limitations. It is a single-center observational study, so it was difficult to avoid potential remains of residual confounding. Our subgroups were uneven, with smaller number of patients in the pancreatitis subgroup and the trauma subgroup than in the peritonitis subgroup and the trauma with secondary sepsis subgroup. Therefore, trends and patterns in investigated cell counts and ratios that we found should be validated in larger patient population, so further studies are warranted. We cannot generalize our results to other subgroups of critically ill and injured patients. Also, given the fact that the time point of measurement in our study, although in line with most of the other similar studies, might not always coincide with the worst measurement in the first 24 hours, we cannot claim that results would be the same if the worst measurement would always be used for calculation. Another limitation of our study is single measurement of investigated biomarkers.

5. Conclusions

This study demonstrates a clear relationship between higher NLR and MPV/PC levels and lethal outcome in critically ill patients with peritonitis and pancreatitis, whereas MLR and PLR did not differ significantly between survivors and nonsurvivors. Our data showed that there was no significant difference of investigated biomarkers between survivors and nonsurvivors in trauma patients with and without sepsis except for significantly higher PLR in trauma without sepsis nonsurvivors. Independent predictor of lethal outcome was NLR in the whole cohort and in the peritonitis subgroup as well as MPV in the pancreatitis subgroup. For the first time, we demonstrate statistically significant differences in MPV/PC, MLR, and PLR values regarding nature of bacteremia. In general, the lowest levels had been found in patients with Gram-positive blood cultures. In heterogeneous cohort subgroup, analysis is essential. Therefore, trends and patterns in investigated cell counts and ratios that we found should be validated in larger patient population, so further studies are warranted. We cannot generalize our results to other subgroups of critically ill and injured patients.

Data Availability

Reasonable requests for data, up to 12 months after initial publication, will be considered by corresponding author.

Conflicts of Interest

The authors declare that there is no conflict of interests.

Supplementary Materials

Table 1: baseline and laboratory characteristics of the peritonitis subgroup according to outcome. Table 2: clinical accuracy of baseline biomarkers in predicting lethal outcome in the peritonitis subgroup. Table 3: clinical accuracy of baseline biomarkers in predicting negative blood culture in the peritonitis subgroup. Table 4: baseline and laboratory characteristics of the pancreatitis subgroup. Table 5: clinical accuracy of baseline biomarkers in predicting lethal outcome in the pancreatitis subgroup. Table 6: clinical accuracy of baseline biomarkers in predicting negative blood culture in the pancreatitis subgroup. Table 7: baseline and laboratory characteristics of the trauma + sepsis subgroup. Table 8: clinical accuracy of baseline biomarkers in predicting polymicrobial blood culture in trauma patients with secondary sepsis. Table 9: baseline and laboratory characteristics of the trauma subgroup. Table 10: clinical accuracy of baseline biomarker PLR in predicting lethal outcome in the trauma subgroup. Table 11: Spearman rho correlations between biomarkers in all patients. Table 12: Spearman rho correlations between biomarkers in the trauma group. Table 13: Spearman rho correlations between biomarkers in survivors with Gram-positive and negative blood cultures. Table 14: Spearman rho correlations between biomarkers in nonsurvivors with Gram-positive and polymicrobial blood cultures. Table 15: univariate odds ratios of variables for predicting lethal outcome in the peritonitis subgroup. Table 16: independent predictors of lethal outcome by multivariate logistic regression analysis in the peritonitis subgroup. Table 17: univariate odds ratios of variables for predicting lethal outcome in the pancreatitis subgroup. Table 18: independent predictors of lethal outcome by multivariate logistic regression analysis in the pancreatitis subgroup. Figure 1: receiver operating characteristic (ROC) curve for composite bioscore in the peritonitis subgroup and the lethal outcome. Figure 2: receiver operating characteristic (ROC) curve for composite bioscore in the trauma with secondary sepsis subgroup and the lethal outcome. Figure 3: receiver operating characteristic (ROC) curve for composite bioscore in the trauma subgroup and the lethal outcome. Figure 4: scattergram for the trauma subgroup (survivors and nonsurvivors) of: A. MPV/PC versus MLR; B. NLR versus MLR; C. MPV/PC versus PLR; D. MLR versus PLR. *(Supplementary Materials)*

References

[1] M. Surbatovic, M. Veljovic, J. Jevdjic, N. Popovic, D. Djordjevic, and S. Radakovic, "Immunoinflammatory response in critically ill patients: severe sepsis and/or trauma," *Mediators of Inflammation*, vol. 2013, Article ID 362793, 11 pages, 2013.

[2] D. Djordjevic, J. Pejovic, M. Surbatovic et al., "Prognostic value and daily trend of interleukin-6, neutrophil CD64 expression, C-reactive protein and lipopolysaccharide-binding protein in critically ill patients: reliable predictors of outcome or not?," *Journal of Medical Biochemistry*, vol. 34, no. 4, pp. 431–439, 2015.

[3] X. Liu, Y. Shen, H. Wang, Q. Ge, A. Fei, and S. Pan, "Prognostic significance of neutrophil-to-lymphocyte ratio in patients with sepsis: a prospective observational study," *Mediators of Inflammation*, vol. 2016, Article ID 8191254, 8 pages, 2016.

[4] A. Naess, S. S. Nilssen, R. Mo, G. E. Eide, and H. Sjursen, "Role of neutrophil to lymphocyte and monocyte to lymphocyte ratios in the diagnosis of bacterial infection in patients with fever," *Infection*, vol. 45, no. 3, pp. 299–307, 2017.

[5] C.-F. Zheng, W.-Y. Liu, F.-F. Zeng et al., "Prognostic value of platelet-to-lymphocyte ratios among critically ill patients with acute kidney injury," *Critical Care*, vol. 21, no. 1, p. 238, 2017.

[6] G. H. Oh, S. P. Chung, Y. S. Park et al., "Mean platelet volume to platelet count ratio as a promising predictor of early mortality in severe sepsis," *Shock*, vol. 47, no. 3, pp. 323–330, 2017.

[7] M. Singer, C. S. Deutschman, C. W. Seymour et al., "The third international consensus definitions for sepsis and septic shock (Sepsis-3)," *JAMA*, vol. 315, no. 8, pp. 801–810, 2016.

[8] J. D. Salciccioli, D. C. Marshall, M. Pimentel et al., "The association between the neutrophil-to-lymphocyte ratio and mortality in critical illness: an observational cohort study," *Critical Care*, vol. 19, no. 1, p. 13, 2015.

[9] B. Azab, N. Jaglall, J. P. Atallah et al., "Neutrophil-lymphocyte ratio as a predictor of adverse outcomes of acute pancreatitis," *Pancreatology*, vol. 11, no. 4, pp. 445–452, 2011.

[10] R. Terradas, S. Grau, J. Blanch et al., "Eosinophil count and neutrophil-lymphocyte count ratio as prognostic markers in patients with bacteremia: a retrospective cohort study," *PLoS One*, vol. 7, no. 8, article e42860, 2012.

[11] F. Riché, E. Gayat, R. Barthélémy, M. le Dorze, J. Matéo, and D. Payen, "Reversal of neutrophil-to-lymphocyte count ratio in early versus late death from septic shock," *Critical Care*, vol. 19, no. 1, p. 439, 2015.

[12] P. G. Vaughan-Shaw, J. R. E. Rees, and A. T. King, "Neutrophil lymphocyte ratio in outcome prediction after emergency abdominal surgery in the elderly," *International Journal of Surgery*, vol. 10, no. 3, pp. 157–162, 2012.

[13] E. Dilektasli, K. Inaba, T. Haltmeier et al., "The impact of neutrophil-to-lymphocyte ratio on mortality in critically ill trauma patients," *Journal of the American College of Surgeons*, vol. 221, no. 4, article S168, 2015Supplement 1, 2015.

[14] C. P. C. de Jager, P. C. Wever, E. F. A. Gemen et al., "The neutrophil-lymphocyte count ratio in patients with community-acquired pneumonia," *PLoS One*, vol. 7, no. 10, article e46561, 2012.

[15] C. P. C. de Jager, P. T. L. van Wijk, R. B. Mathoera, J. de Jongh-Leuvenink, T. van der Poll, and P. C. Wever, "Lymphocytopenia and neutrophil-lymphocyte count ratio predict bacteremia better than conventional infection markers in an emergency care unit," *Critical Care*, vol. 14, no. 5, article R192, 2010.

[16] H. B. Zhang, J. Chen, Q. F. Lan, X. J. Ma, and S. Y. Zhang, "Diagnostic values of red cell distribution width, platelet distribution width and neutrophil-lymphocyte count ratio for sepsis," *Experimental and Therapeutic Medicine*, vol. 12, no. 4, pp. 2215–2219, 2016.

[17] G. Gurol, I. H. Ciftci, H. A. Terzi, A. R. Atasoy, A. Ozbek, and M. Koroglu, "Are there standardized cutoff values for

neutrophil-lymphocyte ratios in bacteremia or sepsis?," *Journal of Microbiology and Biotechnology*, vol. 25, no. 4, pp. 521–525, 2015.

[18] J. Wang, Y. Yin, X. Wang et al., "Ratio of monocytes to lymphocytes in peripheral blood in patients diagnosed with active tuberculosis," *The Brazilian Journal of Infectious Diseases*, vol. 19, no. 2, pp. 125–131, 2015.

[19] J. Xiang, L. Zhou, X. Li et al., "Preoperative monocyte-to-lymphocyte ratio in peripheral blood predicts stages, metastasis, and histological grades in patients with ovarian cancer," *Translational Oncology*, vol. 10, no. 1, pp. 33–39, 2017.

[20] W. K. Eo, S. Kwon, S. B. Koh et al., "The lymphocyte-monocyte ratio predicts patient survival and aggressiveness of endometrial cancer," *Journal of Cancer*, vol. 7, no. 5, pp. 538–545, 2016.

[21] E. Emektar, S. K. Corbacioglu, S. Dagar, H. Uzunosmanoglu, T. Safak, and Y. Cevik, "Prognostic value of the neutrophil–lymphocyte and platelet–lymphocyte ratios in predicting one-year mortality in patients with hip fractures and aged over 60 years," *Eurasian Journal of Emergency Medicine*, vol. 16, no. 4, pp. 165–170, 2017.

[22] M. Bekdas and H. Ozturk, "Platelet-to-lymphocyte ratio in diagnosis of acute complicated appendicitis in childhood," *Journal of Clinical and Analytical Medicine*, vol. 8, no. 4, pp. 299–301, 2017.

[23] Y. Furuncuoğlu, S. Tulgar, A. N. Dogan, S. Cakar, Y. K. Tulgar, and B. Cakiroglu, "How obesity affects the neutrophil/lymphocyte and platelet/lymphocyte ratio, systemic immune-inflammatory index and platelet indices: a retrospective study," *European Review of Medical and Pharmacological Sciences*, vol. 20, no. 7, pp. 1300–1306, 2016.

[24] Y. Shimoyama, O. Umegaki, T. Agui, N. Kadono, and T. Minami, "Neutrophil to lymphocyte ratio and platelet to lymphocyte ratio are superior to other inflammation-based prognostic scores in predicting the mortality of patients with gastrointestinal perforation," *JA Clinical Reports*, vol. 3, no. 1, p. 49, 2017.

[25] Y. Gao, Y. Li, X. Yu et al., "The impact of various platelet indices as prognostic markers of septic shock," *PLoS One*, vol. 9, no. 8, article e103761, 2014.

[26] C. H. Kim, S. J. Kim, M. J. Lee et al., "An increase in mean platelet volume from baseline is associated with mortality in patients with severe sepsis or septic shock," *PLoS One*, vol. 10, no. 3, article e0119437, 2015.

[27] F. G. Zampieri, O. T. Ranzani, V. Sabatoski et al., "An increase in mean platelet volume after admission is associated with higher mortality in critically ill patients," *Annals of Intensive Care*, vol. 4, no. 1, p. 20, 2014.

[28] F. Sadaka, P. L. Donnelly, M. T. Griffin, J. O'Brien, and R. Lakshmanan, "Mean platelet volume is not a useful predictor of mortality in septic shock," *Journal of Blood Disorders & Transfusion*, vol. 5, no. 2, 2014.

[29] E. Guclu, Y. Durmaz, and O. Karabay, "Effect of severe sepsis on platelet count and their indices," *African Health Sciences*, vol. 13, no. 2, pp. 333–338, 2013.

[30] S. Ates, H. Oksuz, B. Dogu, F. Bozkus, H. Ucmak, and F. Yanıt, "Can mean platelet volume and mean platelet volume/platelet count ratio be used as a diagnostic marker for sepsis and systemic inflammatory response syndrome?," *Saudi Medical Journal*, vol. 36, no. 10, pp. 1186–1190, 2015.

[31] M. Legrand, E. Klijn, D. Payen, and C. Ince, "The response of the host microcirculation to bacterial sepsis: does the pathogen matter?," *Journal of Molecular Medicine*, vol. 88, no. 2, pp. 127–133, 2010.

[32] S. Skovbjerg, A. Martner, L. Hynsjö et al., "Gram-positive and Gram-negative bacteria induce different patterns of cytokine production in human mononuclear cells irrespective of taxonomic relatedness," *Journal of Interferon & Cytokine Research*, vol. 30, no. 1, pp. 23–32, 2010.

[33] J. Cohen, "A role for the micro-organism in the outcome from infection? A principle challenged," *Critical Care Medicine*, vol. 39, no. 8, pp. 2001-2002, 2011.

[34] S. J. Finney, S. K. Leaver, T. W. Evans, and A. Burke-Gaffney, "Differences in lipopolysaccharide- and lipoteichoic acid-induced cytokine/chemokine expression," *Intensive Care Medicine*, vol. 38, no. 2, pp. 324–332, 2012.

[35] X. J. Xu, Y. M. Tang, C. Liao et al., "Inflammatory cytokine measurement quickly discriminates gram-negative from gram-positive bacteremia in pediatric hematology/oncology patients with septic shock," *Intensive Care Medicine*, vol. 39, no. 2, pp. 319–326, 2013.

[36] H. Janols, C. Bergenfelz, R. Allaoui et al., "A high frequency of MDSCs in sepsis patients, with the granulocytic subtype dominating in gram-positive cases," *Journal of Leukocyte Biology*, vol. 96, no. 5, pp. 685–693, 2014.

[37] D. I. Gabrilovich, "Editorial: the intricacy of choice: can bacteria decide what type of myeloid cells to stimulate?," *Journal of Leukocyte Biology*, vol. 96, no. 5, pp. 671–674, 2014.

[38] M. Ost, A. Singh, A. Peschel, R. Mehling, N. Rieber, and D. Hartl, "Myeloid-derived suppressor cells in bacterial infections," *Frontiers in Cellular and Infection Microbiology*, vol. 6, p. 37, 2016.

[39] E. Minejima, J. Bensman, R. C. She et al., "A dysregulated balance of proinflammatory and anti-inflammatory host cytokine response early during therapy predicts persistence and mortality in Staphylococcus aureus bacteremia," *Critical Care Medicine*, vol. 44, no. 4, pp. 671–679, 2016.

[40] N. Antonakos, T. Tsaganos, V. Oberle et al., "Decreased cytokine production by mononuclear cells after severe gram-negative infections: early clinical signs and association with final outcome," *Critical Care*, vol. 21, no. 1, p. 48, 2017.

[41] M. Surbatovic, N. Popovic, D. Vojvodic et al., "Cytokine profile in severe Gram-positive and Gram-negative abdominal sepsis," *Scientific Reports*, vol. 5, no. 1, 2015.

[42] H. Aydemir, N. Piskin, D. Akduman, F. Kokturk, and E. Aktas, "Platelet and mean platelet volume kinetics in adult patients with sepsis," *Platelets*, vol. 26, no. 4, pp. 331–335, 2015.

[43] S. Gibot, M. C. Béné, R. Noel et al., "Combination biomarkers to diagnose sepsis in the critically ill patient," *American Journal of Respiratory and Critical Care Medicine*, vol. 186, no. 1, pp. 65–71, 2012.

[44] D. Andaluz-Ojeda, F. Bobillo, V. Iglesias et al., "A combined score of pro- and anti-inflammatory interleukins improves mortality prediction in severe sepsis," *Cytokine*, vol. 57, no. 3, pp. 332–336, 2012.

[45] S. Angeletti, G. Dicuonzo, M. Fioravanti et al., "Procalcitonin, MR-proadrenomedullin, and cytokines measurement in sepsis diagnosis: advantages from test combination," *Disease Markers*, vol. 2015, Article ID 951532, 14 pages, 2015.

[46] L. Ljungström, A.-K. Pernestig, G. Jacobsson, R. Andersson, B. Usener, and D. Tilevik, "Diagnostic accuracy of procalcitonin, neutrophil-lymphocyte count ratio, C-reactive protein, and lactate in patients with suspected bacterial sepsis," *PLoS One*, vol. 12, no. 7, article e0181704, 2017.

[47] N. Stankovic, I. Stanojevic, D. Djordjevic et al., "Neutrophil-to-lymphocyte ratio in pediatric acute appendicitis," *Vojnosanitetski Pregled*, vol. 75, no. 1, pp. 46–55, 2018.

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Research Article

Sophocarpine Attenuates LPS-Induced Liver Injury and Improves Survival of Mice through Suppressing Oxidative Stress, Inflammation, and Apoptosis

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Septic liver injury/failure that is mainly characterized by oxidative stress, inflammation, and apoptosis led to a great part of terminal liver pathology with limited effective intervention. Here, we used a lipopolysaccharide (LPS) stimulation model to simulate the septic liver injury and investigated the effect of sophocarpine on LPS-stimulated mice with endotoxemia. We found that sophocarpine increases the survival rate of mice and attenuates the LPS-induced liver injury, which is indicated by pathology and serum liver enzymes. Further research found that sophocarpine ameliorated hepatic oxidative stress indicators (H_2O_2, $O_2^{\bullet -}$, and NO) and enhanced the expression of antioxidant molecules such as superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH). In addition, sophocarpine also attenuated regional and systematic inflammation and further reduced apoptosis of hepatocytes. Mechanistic evidence was also investigated in the present study as sophocarpine inhibited hepatic expression of the CYP2E/Nrf2 pathway during oxidative stress, inactivated p38/JNK cascade and NF-κB pathway, and, meanwhile, suppressed PI3K/AKT signaling that reduced apoptosis. Conclusively, the present study unveiled the protective role of sophocarpine in LPS-stimulated oxidative reaction, inflammation, and apoptosis by suppressing the CYP2E/Nrf2/ROS as well as PI3K/AKT pathways, suggesting its promising role in attenuating inflammation and liver injury of septic endotoxemia.

1. Introduction

The liver plays a key role in immunological homeostasis and metabolism [1] while these crucial functions are usually impaired by lipopolysaccharide (LPS), inflammatory factors, and pathogens [2, 3]. LPS presents the major component of endotoxin in gram-negative bacteria and causes uncontrolled production of inflammatory mediators and oxidative stress, resulting in acute liver injury (and failure) [4]. LPS-induced liver injury in mice has been employed as a model for molecular pathological research [5], simulating the course of liver damage and failure in septic endotoxemia or sometimes septic shock or death [2, 6]. Liver failure is characterized by hepatic encephalopathy and disorder of protein synthesis [6]. Though specific mechanism remains controversial, consensus has been well reached that hepatic inflammatory oxidative stress and apoptosis might be the crucial mechanism.

Sepsis progression and septic liver dysfunction present complex pathophysiological alterations, [7], including processes like releasing of reactive oxygen species (ROS), nitrogen species (RNS), inflammation, and apoptosis. Characterized by the imbalance of endogenous enzymatic activity, such as catalase (CAT), superoxide dismutase (SOD), and glutathione (GSH) [8, 9], oxidative damage could be reflected by CYP2E1, which promotes the production of ROS during its catalytic cycle and may be the main contributor to oxidative stress and liver injury [10–12]. Thus, antioxidant compounds have been considered a promising treatment against ROS-induced liver injury or failure [13].

Additionally, oxidative stress is able to activate the p38 MAPK pathway, resulting in the activation of mitochondrial-related or other apoptotic pathways [14, 15]. In addition to the well acknowledged notion that phosphatidylinositol 3 kinase (PI3K)/Akt signaling as a key modulator of apoptotic process [16, 17], recent research proposed AKT could regulate apoptosis-related proteins including Bax, caspase-9, and caspase-3, which are also crucially involved in anticancer drug-stimulated apoptosis of cancer cells [18]. Nevertheless, the potential role of PI3K/AKT in liver injury remains unclear.

Liver injury induced by LPS is associated with inflammatory mediators including superoxide, nitric oxide, and tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and other cytokines [19, 20]. LPS may activate the transcription factor nuclear factor-kappa B (NF-κB) leading to activation of many inflammatory genes, such as TNF-α and IL-1β [19–21]. Therefore, inactivation of NF-κB could attenuate LPS-induced sepsis and liver injury or liver failure.

Sophora alkaloids, such as sophocarpine, are able to be separated from the traditional Chinese herbs, for example, *Sophora flavescens* and *Sophora alopecuroides* [22, 23]. It has been reported that the extracts of *Sophora alopecuroides*, named Kudouzhi injection in clinical, have been used for the treatment of inflammation, pain, edema, and fever [23]. Furthermore, sophocarpine, called Kangke injection in clinical, has been employed to inhibit viral replication [24]. Sophocarpine also shows its activity against inflammation [23], and it suppresses inflammatory processes by inhibiting the JNK pathway, p38 MAPK pathway, and NF-κB pathway, reducing the levels of iNOS and COX-2 [25]. However, the mechanisms underlying the role of sophocarpine remains unknown in LPS-induced liver injury.

In the present study, sophocarpine improved the liver function and protected the liver from inflammatory liver injury. To investigate its molecular mechanisms, the role of NF-κB, PI3K/AKT, and CYP2E/Nrf2/ROS had been determined in acute liver injury. In the end, we evaluated the effects of sophocarpine on oxidative stress-associated apoptosis and inflammation in LPS-induced mice.

2. Materials and Methods

2.1. Cell Culture and Reagents. The hepatic stellate cells (HSCs) were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in DMEM media supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco, Waltham, MA, USA) and 10% fetal bovine serum (FBS) (Gibco, Waltham, MA, USA) in a humidified incubator containing 5% CO_2 and 95% air at 37°C. The HSCs were incubated with LPS (100 ng/mL) in the absence or presence of sophocarpine at the concentration of 1 μM and 2 μM about 24 h. These cells were used for the further studies.

2.2. Animals. Eight-week-old C57 BL/6 male mice, about 22.1 g per mouse, were employed for further study. And these mice were bought from the Experimental Animal Centre of Second Military Medical University (Shanghai, China) and were approved by the Animal Care and Use Committee of Changhai Hospital, Second Military Medical University (Shanghai, China). They were placed in a specific pathogen-free (SPF) room with sawdust bedding at a temperature of 25-26°C and a relative humidity of ~50% and light 12 h/day, and water and food were free to access. The authors confirmed that all animals received human care, and all animal experiments were conducted in accordance with the relevant guidelines and regulations. In this study, the normal mice were starved about 16 hours and they were divided randomly into four groups: con (control), LPS-induced group, and LPS-induced mice pretreated with sophocarpine (30 mg/kg body weight per day and 60 mg/kg body weight per day). For the establishment of liver injury in LPS-administrated mice with endotoxemia, the mice were injected intraperitoneally with LPS (5 mg/kg body weight) and we prepared 30 mice for each group above. Sophocarpine was administered orally once at 16:00~17:00 every day for 24 days. Meanwhile, we marked the number of dead mice for each group.

2.3. Analyses of Liver Function. Performed as the indicators of hepatic function, serum and liver levels of glutathione (GSH), alanine transaminase (ALT), alkaline phosphatase (ALP), and aspartate transaminase (AST) were analyzed by employing the biochemical kits from R&D Systems (Minneapolis, MN, USA).

2.4. Analyses of H_2O_2 and $O_2^{\bullet-}$ Production and ROS Levels in Liver of Mice. Hepatic levels of $O_2^{\bullet-}$ were measured using the chemiluminescence method [26]. Firstly, the weighed liver tissues of mice were homogenized in lysis buffer, pH 7.4, containing 10 mM EDTA as well as 20 mM HEPES. The samples were centrifuged for 10 min at 1000g, and, then, the aliquot of samples was incubated with a Krebs-HEPES buffer, pH 7.4, containing 5 mM lucigenin (Sigma, Shanghai, China) about 2 min at 37°C. Next, light emission data were obtained on a M200 PRO multifunctional microplate reader (TECAN, Switzerland), and the results were showed as mean light unit (MLU) min/mg protein. Levels of $O_2^{\bullet-}$ were measured by adding SOD (350 U/mL) to the medium according to the manufacturer's instruction (R&D Systems, Minneapolis, MN, USA). In addition, liver tissues were homogenized in normal saline, and the samples were treated with equal volume of cold methanol for 60 min in a 4°C icebox. Then, the samples were centrifuged for half an hour at 10000g and we obtained the supernatant for H_2O_2 evaluation using the biochemical kits from the R&D Systems (Minneapolis, MN, USA). Protein concentration was measured using the Bradford method, and BSA was employed as the standard.

2.5. Determination of IL-1β, TNF-α, and IL-16 by ELISA. The weighed liver tissues were put in a cold PBS buffer (pH 7.0) containing 0.002% sodium acid, 0.1 mg/mL soybean trypsin inhibitor, 2 mM PMSF, 10 nM EDTA, and 1.0 mg/mL BSA. The tissues were homogenated, and, then, the samples were incubated for 2 h in a 4°C refrigerator. For further assays, the supernatants were collected by centrifugation at 12000g for 10 min. IL-1β, TNF-α, and IL-16 levels in the supernatant of the serum and liver were measured using ELISA kits (Sigma, Shanghai, China).

2.6. Hematoxylin-Eosin Staining. The liver tissues from mice were fixed in 10% formalin, and the fixed specimens were processed to paraffin blocks, sectioned (5 μm), and stained with hematoxylin-eosin (H&E) for histological analysis according to the standard protocols [27]. In this study, the sections were observed in a blind manner [27].

2.7. Reverse Transcription Polymerase Chain Reaction (RT-PCR). The reverse transcription polymerase chain reaction (RT-PCR) and the quantitative real-time PCR (Q-PCR) were performed as previously described [20]. Total RNA was extracted from liver tissues and HSCs using TRIzol reagent from Thermo Fisher Scientific (Waltham, MA, USA). The cDNA was obtained by reverse transcription in a 20 μL reaction containing 2 μg of total RNA, oligo (dT), and reverse transcription premix.

The quantitative real-time PCR (Q-PCR) reactions were performed with the SYBR green PCR system in an ABI 7500 thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). The SYBR green reagents were also purchased from Thermo Fisher Scientific. The cycling conditions were as follows: 95°C for 3 min; followed by 40 cycles involving denaturing at 95°C for 10 s, annealing at 60°C for 5 s, and extension at 72°C for 10 s. Expression of mRNAs was normalized by the mRNA levels of β-actin, which was used as an internal control. The primers were shown here are PI3K, sense, 5′-TGACAGTAGGAGGAGGTTGG-3′, antisense, 5′-TCAGCCACATCAAGTATTGG-3′; AKT, sense, 5′-GAAGGTGATTCTGGTGAAAGAG-3′, antisense, 5′-ACACGGTTCTCAGTAAGCG-3′; caspase-9, sense, 5′-TCTTCATCTCCTGCTTAGAGG-3′, antisense, 5′-TGCTCCTTTGCTGTGAGTC-3′; caspase-3, sense, 5′-TGGAAAGCCGAAACTCTTC-3′, antisense, 5′-AGGAATAGTAACCAGGTGCTG-3′; caspase-6, sense, 5′-AGCGCGTACTTAAATGCAGAGG-3′, antisense, 5′-GTTGTAAGGTGGACAGGCTT-3′; Cyto-C, sense, 5′-CAGACAAGAAGAGGTTGCC-3′, antisense, 5′-CGTCATGGCAGTGTGTATTGG-3′; Bad, sense, 5′-CAGAGTTTGAGCCGAGTGAG-3′, antisense, 5′-TCCCTGCTGATGAATGTTG-3′; Bcl-xL, sense, 5′-GCAGGCGATGAGTTTGAAC-3′, antisense, 5′-TCCTTGTCTACGCTTTCCAC-3′; GAPDH, sense, 5′-CATTCAAGACCGGACAGAGG-3′, antisense, 5′-ACATACTGCACACCAGCATCACC-3′. In the end, we analyzed the relative levels of mRNAs using the $2^{-\Delta\Delta Ct}$ method and GAPDH was considered as the internal control.

2.8. Immunoblot Analysis. The liver tissues or the HSCs were lysed in RIPA Buffer (1 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0, 2% SDS, and 5 mM DTT), and their protein concentration was decided by the BCA assay (Beyotime Inc., Shanghai, China). The total protein (about 30 μg) was separated by a SDS-PAGE gel and transferred to PVDF (polyvinylidene fluoride) membranes (Invitrogen, CA, USA)= and blocked with 5% nonfat dry milk in PBST (phosphate-buffered saline with Tween), pH 7.5. The membranes were immunoblotted with primary antibodies for 4 hours or overnight at 4°C. The primary antibodies were all purchased from Cell Signaling Technology (MA, USA), and they were diluted at 1 : 1000 in the immunoblot analysis. Secondary antibodies with horseradish peroxidase were used in this study. The protein bands were determined by an enhanced chemiluminescence kit (Pierce, Rockford, USA). The corresponding semiquantitative analysis was based on optical density with ImageJ software.

2.9. Determination of the Apoptotic Cells by TUNEL. We determined the apoptosis of HSCs using TUNEL methods as previously described [19]. Briefly, the TUNEL and DAPI, which were from Sigma (Shanghai, China), were used to detect the apoptosis of cultured cells and the apoptotic cells could be TUNEL-positive. Then, the TUNEL-positive HSCs were calculated under a Carl Zeiss microscope (Axio Observer A1, Jena, Germany).

2.10. Statistical Analysis. Data were shown as the mean ± SEM. Student's *t*-test was performed for comparisons between two groups, and one-way ANOVA test was employed for comparisons among several groups. Log-rank test was used for survival data. P value < 0.05 was considered to be statistically significant.

3. Results

3.1. Sophocarpine Increases the Survival Rate and Attenuates the LPS-Induced Liver Injury. The data in our study suggested that the 16-day survival rate was 73.3% (22 out of 30) and 76.7% (22 out of 30) in sophocarpine-pretreated group in a dose-dependent manner; meanwhile, the 16-day survival rate was 30.0% (9 out of 30) in the sepsis group (Figure 1(a)). Compared to the sepsis group, the 16-day survival rate was higher in the sophocarpine-treated group ($P < 0.001$); in the sham group (30 mice), the survival rate was 100% on the 16th day. In a word, pretreatment of mice with sophocarpine before LPS injection remarkably decreased lethality in contrast to LPS-caused sepsis animals.

It has been reported that LPS-induced liver dysfunction may be assessed by serum liver-specific enzymes including AST, ALT, and ALP, and the morphological alterations of the liver may be observed by H&E staining. Firstly, we found that sophocarpine (30 mg/kg and 60 mg/kg per day) recovered destructive damage of hepatocytes significantly in LPS-induced septic liver injury (Figures 1(b) and 1(c)). Then, AST, ALT, and ALP levels in sepsis mice were higher than sham (normal) group, and sophocarpine significantly decreased AST, ALT, and ALP levels in the serum and liver of sepsis mice (Figures 2(a)–2(f)). Combined with the survival rate in Figure 1(a), the data revealed that sophocarpine showed its protective role in sepsis and sepsis-related acute liver injury via downregulating ALT, AST, and ALP expression.

3.2. Sophocarpine Ameliorates Oxidative Stress-Associated Indicators in LPS-Induced Mice. To demonstrate the effects of sophocarpine on oxidative stress in the liver of LPS-induced mice, we analyzed the antioxidative factors such as SOD, GSH, and CAT and detected the levels of H_2O_2, $O_2^{\bullet -}$, and NO in the liver. The results showed that activity of SOD,

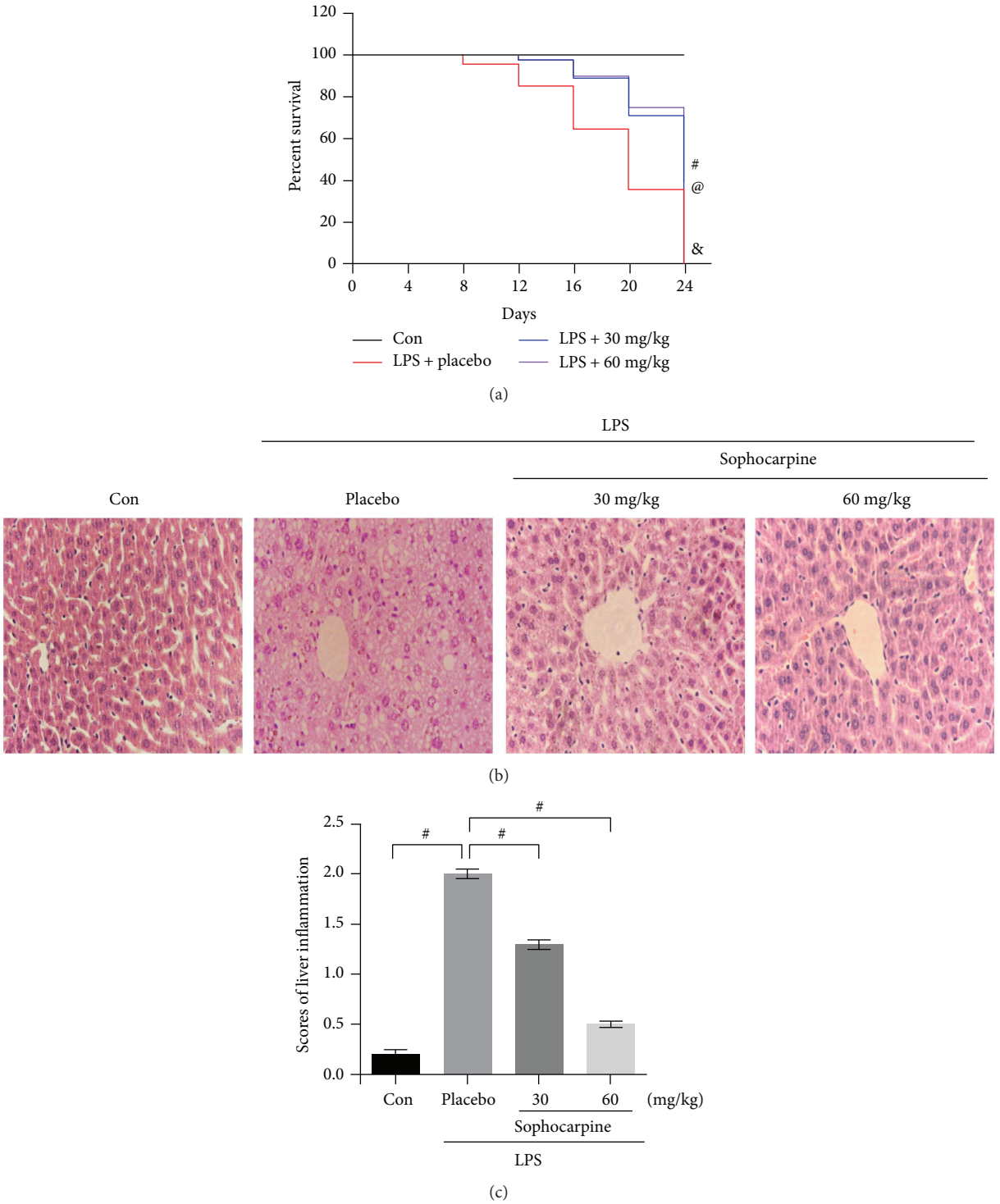

Figure 1: Sophocarpine ameliorates LPS-induced liver injury of mice. (a) Sophocarpine increased survival of endotoxemic mice induced by LPS; $^{\#}P < 0.001$, the sophocarpine-treated group (30 mg/kg body weight per day) versus the LPS-induced group; $^{@}P < 0.001$, the sophocarpine-treated group (30 mg/kg body weight per day) versus the LPS-induced group; $^{\&}P < 0.001$, the LPS-induced group (5 mg/kg body weight) versus the normal group. (b) Liver sections stained with H&E at a magnification of 200x. (c) Assessment of liver injury following portal inflammation scores; data are expressed as mean ± SEM, $n = 30$. $^{\#}P < 0.001$.

CAT, and GSH was decreased obviously in sepsis mice. As expected, sophocarpine (30 and 60 mg/kg) evidently restored CAT activity (Figure 2(g)), GSH activity (Figure 2(h)), and SOD activity (Figure 2(i)). Moreover, hepatic H_2O_2, $O_2^{\bullet -}$, and NO levels were determined in mice. The results indicated that LPS elevated the H_2O_2, $O_2^{\bullet -}$, and NO levels in the liver of mice, and sophocarpine significantly may suppress the H_2O_2, $O_2^{\bullet -}$, and NO production in the liver of LPS-

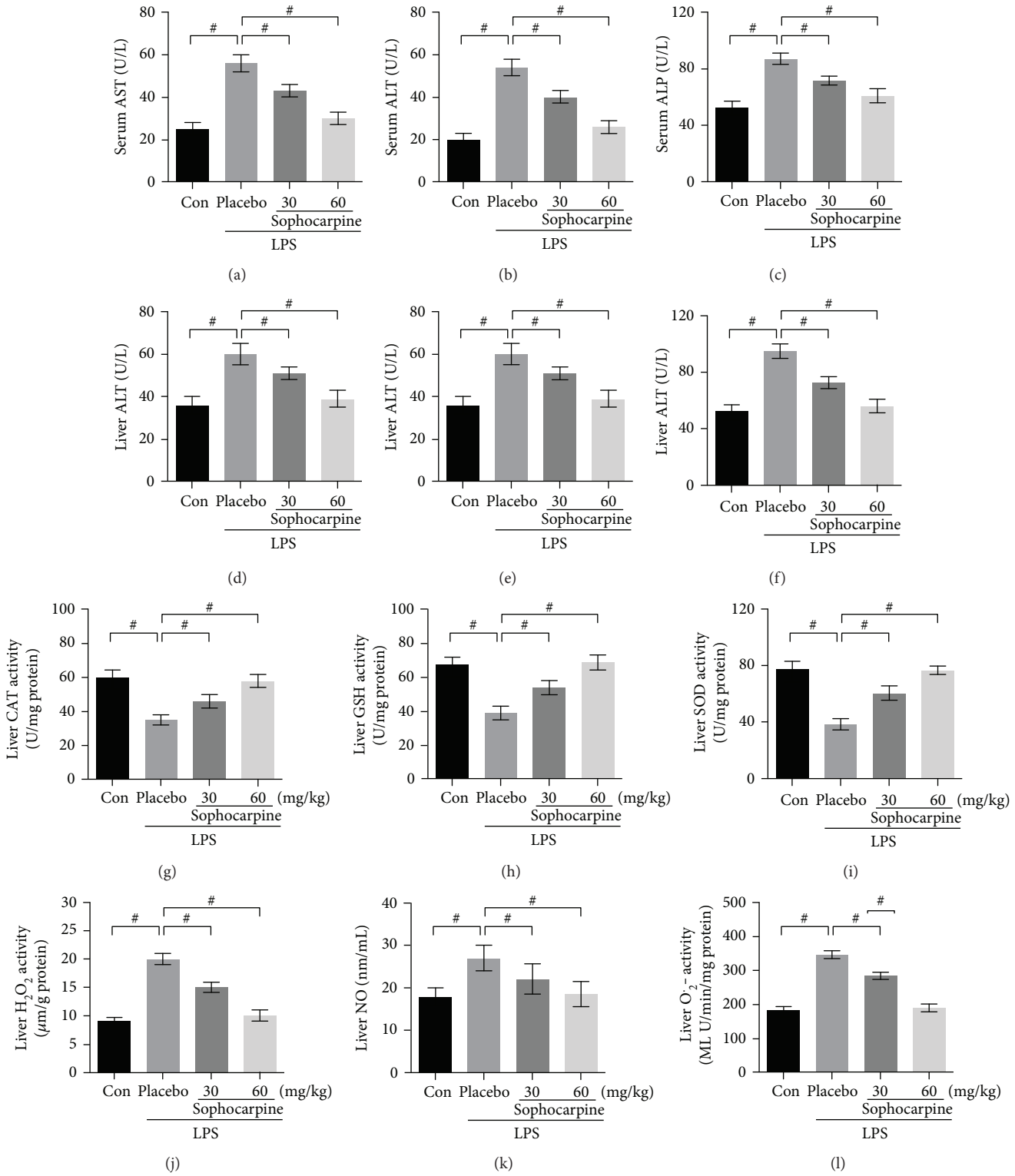

FIGURE 2: Sophocarpine reduces production of liver injury-related factors and improves oxidative stress in the liver of LPS-induced mice. (a–c) Sophocarpine decreased concentrations of AST, ALT, and ALP in serum determined by ELISA. (d–f) Sophocarpine downregulated levels of AST, ALT, and ALP in the liver analyzed by ELISA. The levels of CAT (g), GSH (h), SOD (i), H_2O_2 (j), NO (k), and $O_2^{\cdot-}$ (l) determined by ELISA. Data are expressed as mean ± SEM, $n = 30$. $^{\#}P < 0.001$.

administrated mice (Figures 2(j)–2(l)). Thus, sophocarpine prevented liver injury via attenuating ROS production and oxidative stress in LPS-induced mice.

3.3. The Effects of Sophocarpine on ROS Pathway in LPS-Induced Mice. To investigate the inhibitory mechanism of sophocarpine against oxidative stress, we examined the

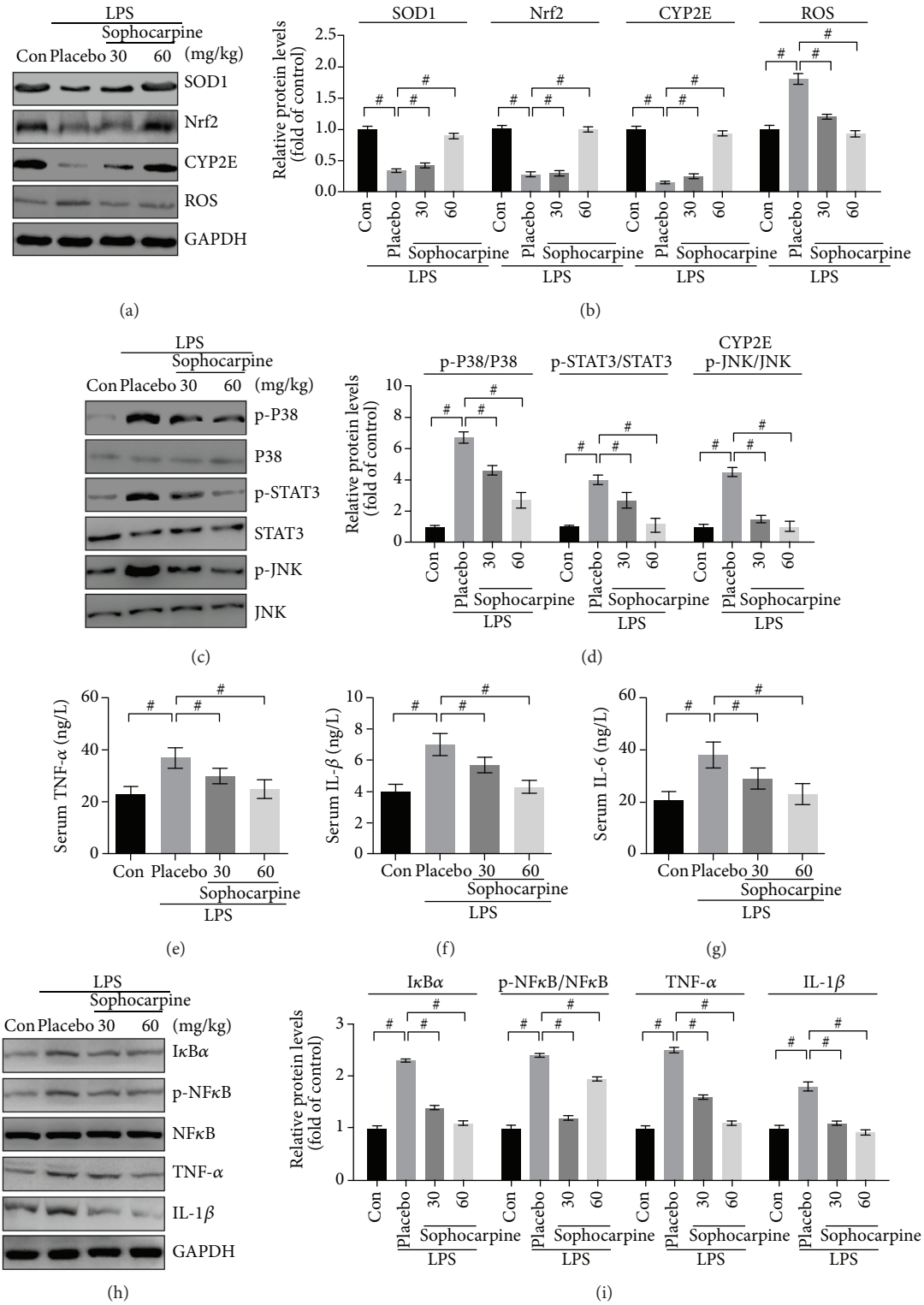

Figure 3: Effect of Sophocarpine on the expression of the hepatic oxidative stress-associated protein and inflammatory signaling pathway in the liver of LPS-induced mice. (a and b) Sophocarpine upregulated the expression of SOD1, Nrf2, and CYPE2 and downregulated the levels of ROS protein detected by Western blot (a) and the semiquantitative analysis of lanes was based on optical density with ImageJ software (b); data are expressed as mean ± SEM, $^{\#}P < 0.001$. (c and d) Sophocarpine inhibited phosphorylation of P38, STAT3, and JNK and (c) and the corresponding semiquantitative analysis was based on optical density with ImageJ software (d). (e–g) Serum levels of TNF-α, IL-1β, and IL-6 analyzed by ELISA in mice; data are expressed as mean ± SEM, $^{\#}P < 0.001$. (h and i) Sophocarpine blocked the expression of IκBα and inactivated the NF-κB; also, sophocarpine suppressed the expression of TNF-α and IL-1β demonstrated by Western blot (h). The semiquantitative analysis of lanes was based on optical density with ImageJ software (i); data are expressed as mean ± SEM, $^{\#}P < 0.001$.

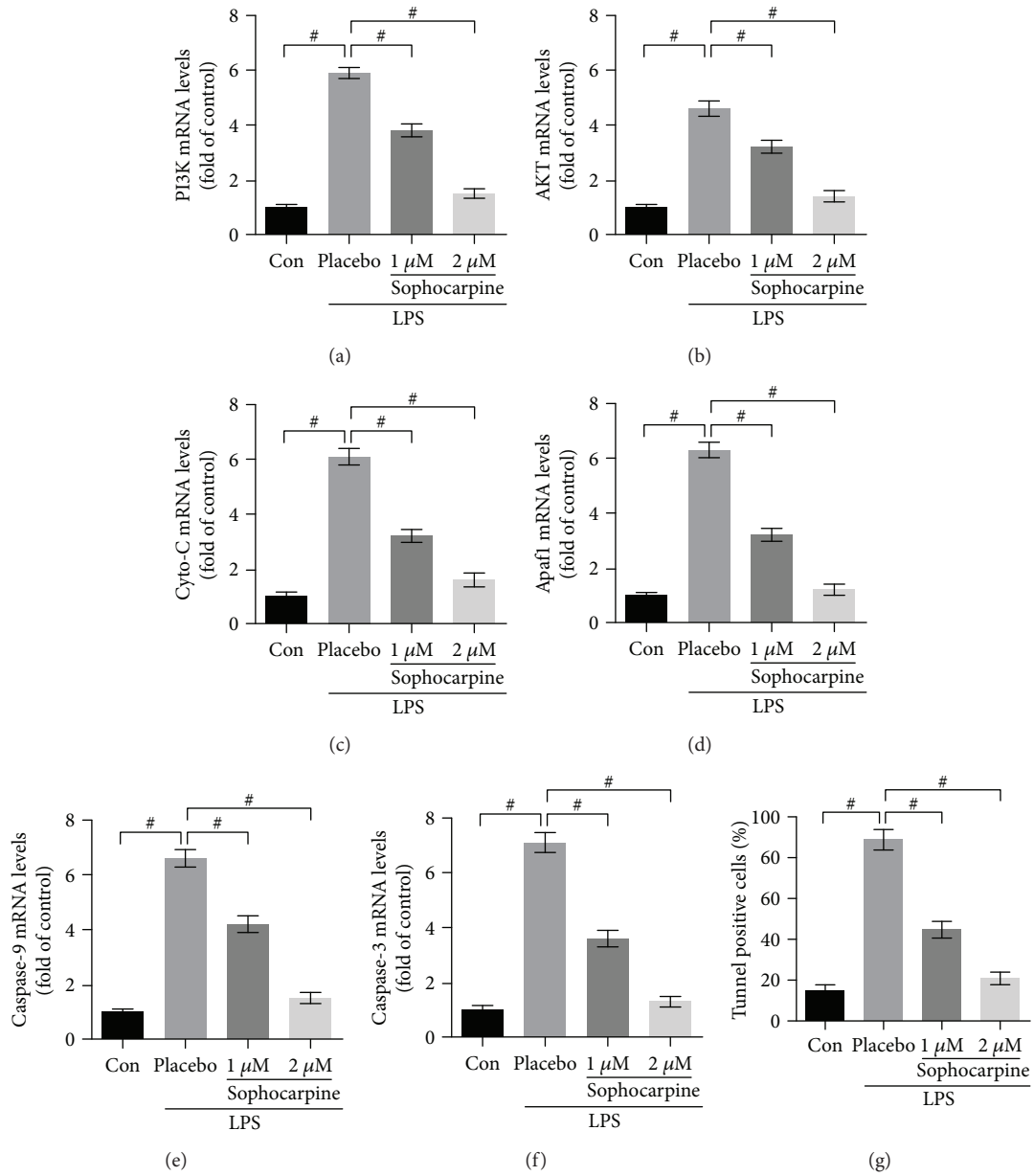

Figure 4: Sophocarpine improves injury of LPS-treated hepatic stellate cells (HSCs) by suppressing apoptosis. (a–f) Sophocarpine decreased the mRNA expression of PI3K, AKT, Cyto-C, Apaf1, caspase-9, and caspase-3 analyzed by real-time PCR; the data are expressed as mean ± SEM, $^{\#}P < 0.001$. (g) Evaluation of apoptosis in HSCs by TUNEL assays and number of TUNEL-positive cells was calculated under a microscope; the data are expressed as mean ± SEM, $^{\#}P < 0.001$.

ROS signaling in the LPS-induced liver of mice by Western blot. As shown in Figure 3, the results demonstrated that SOD1 and Nrf2 expression was dramatically downregulated in the LPS-induced liver, compared with the normal mice. After injection of sophocarpine, data presented that the levels of SOD1 and Nrf2 were elevated markedly by sophocarpine in a dose-dependent manner in endotoxic mice (Figures 3(a) and 3(b)). Moreover, we investigated oxidative stress-associated protein including ROS, CYP2E, P38, JNK, and STAT3 in mice. The data showed that ROS, CYP2E, P38, STAT3, and JNK were increased in LPS-induced mice (Figures 3(c) and 3(d)). However, the expression of CYP2E and ROS, as well as the phosphorylation of P38, STAT3, and JNK, was significantly inhibited by sophocarpine in a dose-dependent manner (Figures 3(c) and 3(d)). Thus, sophocarpine protected against endotoxemia via improving ROS-mediated oxidative stress in the liver of sepsis model animals.

3.4. Sophocarpine Attenuates Inflammation in LPS-Induced Liver Injury. As shown in Figures 3(e)–3(g), the LPS-induced mice exhibited higher content of serum IL-1β, TNF-α, and IL-6 compared with the normal mice. Compared with the LPS-induced group, sophocarpine administration (30 mg/kg and 60 mg/kg per day) significantly reduced serum levels of TNF-α, IL-1β, and IL-6 in LPS-induced mice.

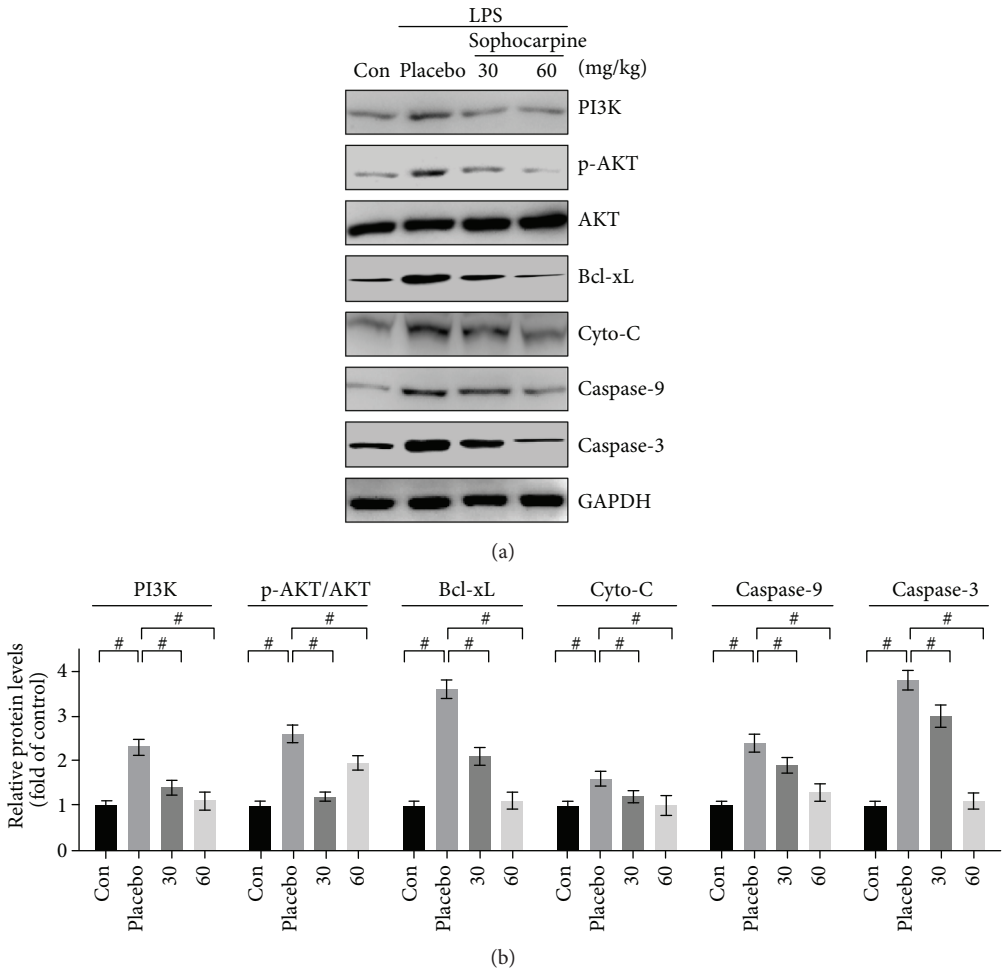

Figure 5: Sophocarpine represses apoptosis-associated signaling protein in the liver of LPS-induced mice. (a) Sophocarpine decreased the expression of PI3K, Bcl-xL, Cyto-C, caspase-9, and caspase-3 and depressed phosphorylation of AKT determined by Western blot. (b) The semiquantitative analysis of lanes was based on optical density with ImageJ software; data are expressed as mean ± SEM, $^{\#}P < 0.001$.

Similarly, we found that LPS enhanced the protein expression of TNF-α and IL-1β, and LPS also upregulated expression of IκBα protein and phosphorylation of NF-κB (Figures 3(h) and 3(i)). Contrarily, sophocarpine downregulated the expression of the above proteins in inflammation of the LPS-induced liver of mice (Figures 3(h) and 3(i)). The data indicated that sophocarpine may ameliorate LPS-induced liver injury by suppressing inflammation responses.

3.5. Sophocarpine Suppresses Apoptosis in Liver of LPS-Induced Mice. To explore the effects of sophocarpine on apoptosis, we analyzed the PI3K/AKT pathway-related apoptosis progressing. The data proved that LPS significantly promoted the expression of PI3K and AKT, which were restored to the normal levels by administration of sophocarpine at 60 mg/kg per day. Furthermore, apoptosis-associated proteins were analyzed by Western blot in this section. Then, we found that Bcl-xL, Cyto-c, Apaf1, and cleaved caspase-9 and caspase-3 were increased by LPS dramatically (Figures 4(a) and 4(b)), indicating that LPS may promote apoptosis development in the liver of mice. After administrating sophocarpine, LPS-induced apoptosis in the liver may be significantly inhibited by depressing the expression of the related proteins above (Figures 5(a) and 5(b)).

To verify the vital role of apoptosis in the progression of LPS-induced acute liver injury, we analyzed the mRNA expression of the apoptosis-associated genes above by real-time PCR. The results showed that sophocarpine markedly downregulated the mRNA levels of PI3K and AKT (Figures 6(a) and 6(b)), and sophocarpine also reduced the mRNA levels of Bad, Bax-xL, Cyto-c, Apaf1, caspase-9, caspase-3, and caspase-6 in the liver of LPS-caused liver failure (Figures 6(c)–6(i)). Thus, sophocarpine attenuated liver injury by repressing the expression of apoptosis-related genes at both mRNA and protein levels.

3.6. Sophocarpine Improves Injury of LPS-Treated HSCs by Suppressing Apoptosis. To study the potential role of sophocarpine on LPS-stimulated hepatic stellate cells (HSCs), we pretreated HSCs with LPS. Then, the LPS-stimulated HSCs were subjected to sophocarpine incubation in order to determine whether sophocarpine may improve the liver injury by regulating apoptosis. In the present study, we found that LPS significantly elevated apoptosis-related gene

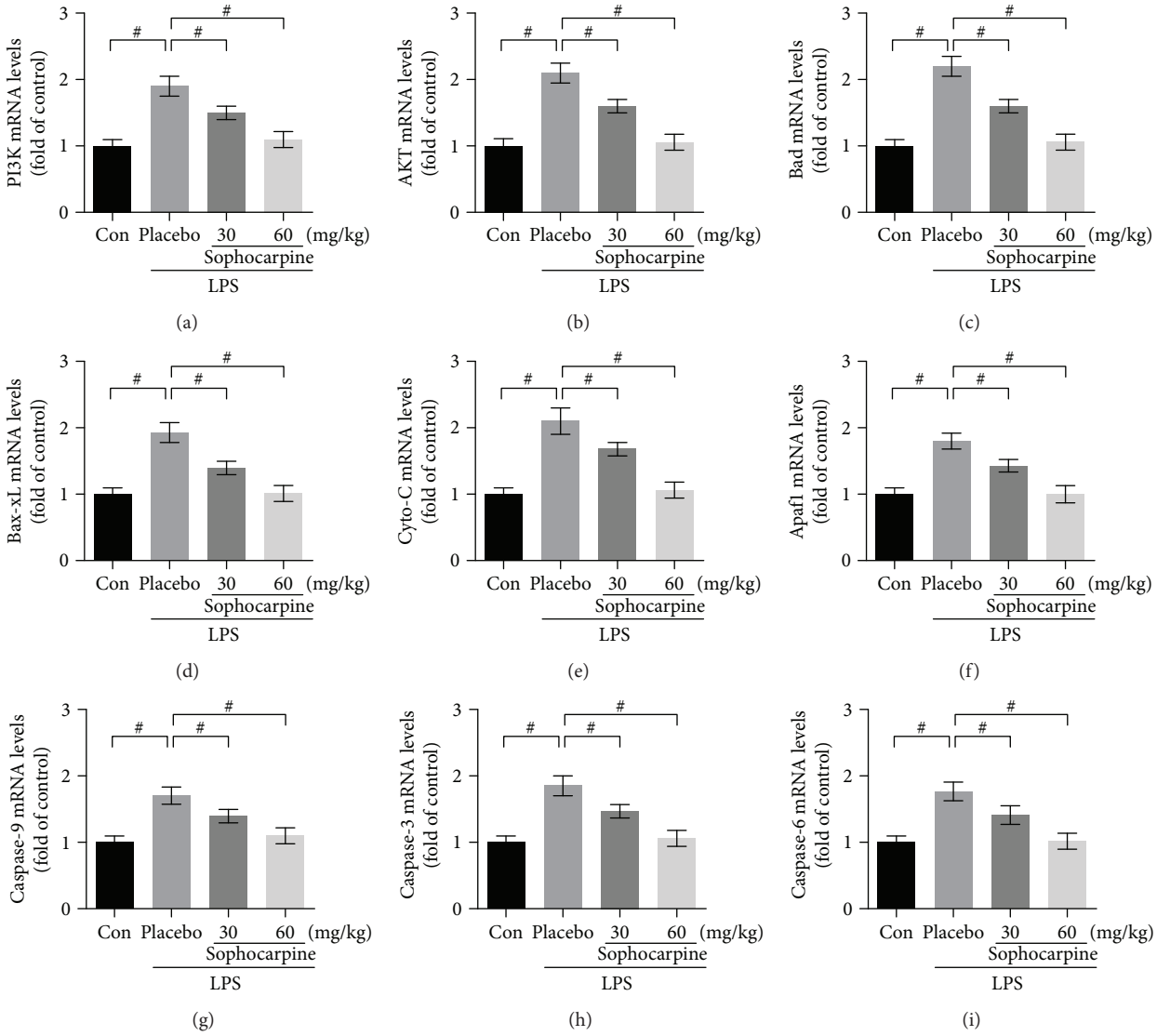

Figure 6: Sophocarpine suppresses apoptosis-associated gene mRNA levels in the liver of LPS-induced mice. Sophocarpine decreased the mRNA expression of PI3K (a), AKT (b), Bad (c), Bcl-xL (d), Cyto-C (e), Apaf1 (f), caspase-9 (g), caspase-3 (h), and caspase-6 (i) analyzed by real-time PCR. The data are expressed as mean ± SEM, $^{\#}P < 0.001$.

expression, including PI3K and AKT, as well as Cyto-C, Apaf1, caspase-9, and caspase-3 (Figures 4(a)–4(f)). However, sophocarpine obviously inhibited this gene expression (Figures 4(a)–4(f)). Subsequently, sophocarpine may attenuate HSC damage and apoptosis in a dose-dependent manner (Figure 4(g)). These in vitro data suggested that sophocarpine could improve HSCs injury by suppressing PI3K/AKT-associated apoptosis.

4. Discussion

Nowadays, the mechanism of acute liver injury (or failure) still has not been completely investigated and required further study for promising clinical strategies [28]. Researches proposed LPS-induced acute liver injury, possibly derived from endotoxemia, was related to the inflammatory-associated Kupffer cells as well as inflammatory mediators including TNF-α, IL-1β, nitric oxide, and superoxide [29]. Furthermore, LPS extends acute liver injury by modulating the oxidative stress and production of free radical, protein synthesis, and apoptosis of hepatocytes [30]. In this study, we found that ALT, AST, and ALP were upregulated in the serum and liver of LPS-induced mice and these three indicators above were dramatically downregulated by sophocarpine (Figures 2(a)–2(f)).

Oxidative stress that is associated with cellular metabolism in the O_2 environment has been regarded as a balance between prooxidant and antioxidant [31]. Based on the cellular microenvironment, the prooxidation process generates ROS including hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\bullet -}$) [32]. The prooxidants H_2O_2, $O_2^{\bullet -}$, and NO are the main sources of ROS production according to the diverse stress conditions [33, 34]. Previous studies have demonstrated that the ROS plays a vital role in septic shock and organ failure [35]. Also, excessive ROS is expressed in LPS-

induced liver injury and antioxidant agents seem to be a good choice to reverse the liver injury [35]. As a main antioxidant and $O_2^{\bullet-}$ scavenger, SOD may react with ROS and NO [36]. CAT may reduce ROS production by degrading H_2O_2 into oxygen and water [37]. Additionally, GSH can protect the liver and other organs against oxidative stress by decreasing the levels of H_2O_2 and lipid hydroperoxide [38]. In the current study, we found that LPS significantly elevated the levels of H_2O_2, $O_2^{\bullet-}$, and NO, which were downregulated nearly to normal levels by sophocarpine in a dose-dependent manner. Meanwhile, sophocarpine obviously upregulated the activity and expression of endogenous antioxidants, such as SOD, CAT, and GSH, suggesting that sophocarpine shows its potential antioxidative by blocking ROS-mediated signaling. As an essential sensor of redox status in ROS process, cellular Nrf2 often binds to the cytoskeletal-anchoring protein under normal conditions [39]. As the main contributor, CYP2E always promotes the production of ROS in liver injury and ethanol-induced oxidative stress [11]. In the present study, LPS decreased the levels of CYP2E, Nrf2, and ROS, which may be restored to normal by sophocarpine administration, indicating that sophocarpine possesses the activity to attenuate oxidative stress resulting in the improvement of LPS-triggered acute liver injury.

It has been reported that PI3K/Akt signaling plays vital roles in survival and antiapoptosis of cells by modulating its downstream targets, including caspase-9, caspase-3, and Bad [14, 34, 36]. Meanwhile, NF-κB signaling has been demonstrated to be an important regulator in apoptosis of cancer cells [34, 36]. However, it remains unknown about the role of PI3K/Akt signaling in the liver of LPS-induced mice. In this study, sophocarpine significantly downregulated the expression of PI3K and phosphorylation of AKT, implying that the chemical can inhibit the expression of activated AKT. Furthermore, we found that sophocarpine reduced the expression of Bcl-xL, which was an antiapoptotic gene. Sophocarpine downregulated the expression of cleaved caspases substantially. Consequently, the data indicate that sophocarpine suppresses the activation of PI3K/AKT signaling resulting in apoptosis of liver injury induced by LPS.

NF-κB signaling is a central regulator of inflammatory cytokines, such as TNF-α and IL-1β, which are key factors in inflammation responses [40]. In the current study, we found that LPS dramatically upregulated content and activity of TNF-α, IL-1β, and IL-6 in the serum and liver of mice. Thus, LPS-induced liver failure may be generated by promoting the inflammation responses including the mentioned inflammatory signaling and cytokines, which can be inhibited by sophocarpine in a dose-dependent manner.

In conclusion, sophocarpine shows the activity against oxidative stress and inflammation in the LPS-induced liver injury of mice. Moreover, sophocarpine suppresses the liver injury in LPS-induced mice with endotoxemia through blocking the inflammatory pathway NF-κB, contributing to downregulation of proinflammatory cytokines. Meanwhile, sophocarpine affects apoptosis in the liver by inhibiting the PI3K/AKT-associated signaling. These findings suggest sophocarpine might be a novel and promising agent to improve inflammation, apoptosis, and oxidative responses in the liver of mice with endotoxemia.

Conflicts of Interest

The authors declare no conflicts of interests.

Authors' Contributions

Zhengyu Jiang and Yan Meng performed the majority of the experiments and contributed to manuscript preparation. Xiaoming Deng and Jinjun Bian designed the whole article and manuscript preparation. Changli Wang and Lulong Bo contributed part of the experiments and analysis of statistics.

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References

[1] J. M. Canabal and D. J. Kramer, "Management of sepsis in patients with liver failure," *Current Opinion in Critical Care*, vol. 14, no. 2, pp. 189–197, 2008.

[2] J. Yan, S. Li, and S. Li, "The role of the liver in sepsis," *International Reviews of Immunology*, vol. 33, no. 6, pp. 498–510, 2014.

[3] T. van der Poll, "Future of sepsis therapies," *Critical Care*, vol. 20, no. 1, p. 106, 2016.

[4] D. M. Boé, T. R. Richens, S. A. Horstmann et al., "Acute and chronic alcohol exposure impair the phagocytosis of apoptotic cells and enhance the pulmonary inflammatory response," *Alcoholism, Clinical & Experimental Research*, vol. 34, no. 10, pp. 1723–1732, 2010.

[5] Z. Zhang, G. J. Bagby, D. Stoltz, P. Oliver, P. O. Schwarzenberger, and J. K. Kolls, "Prolonged ethanol treatment enhances lipopolysaccharide/phorbol myristate acetate-induced tumor necrosis factor-α production in human monocytic cells," *Alcoholism, Clinical & Experimental Research*, vol. 25, no. 3, pp. 444–449, 2001.

[6] W. Bernal, "Acute liver failure: review and update," *International Anesthesiology Clinics*, vol. 55, no. 2, pp. 92–106, 2017.

[7] Y. Hattori, K. Hattori, T. Suzuki, and N. Matsuda, "Recent advances in the pathophysiology and molecular basis of sepsis-associated organ dysfunction: novel therapeutic implications and challenges," *Pharmacology & Therapeutics*, vol. 177, pp. 56–66, 2017.

[8] V. M. Victor, M. Rocha, J. V. Esplugues, and M. De la Fuente, "Role of free radicals in sepsis: antioxidant therapy," *Current Pharmaceutical Design*, vol. 11, no. 24, pp. 3141–3158, 2005.

[9] M. Andrades, C. Ritter, M. R. de Oliveira, E. L. Streck, J. C. Fonseca Moreira, and F. Dal-Pizzol, "Antioxidant treatment reverses organ failure in rat model of sepsis: role of antioxidant enzymes imbalance, neutrophil infiltration, and oxidative stress," *The Journal of Surgical Research*, vol. 167, no. 2, pp. E307–E313, 2011.

[10] A. J. Hayes and D. J. Mole, "Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibition during sepsis dampens the early systemic inflammatory response," *Critical Care Medicine*, vol. 44, no. 8, pp. 1628-1629, 2016.

[11] N. N. Rahbari, A. Mehrabi, N. M. Mollberg et al., "Hepatocellular carcinoma current management and perspectives for the future," *Annals of Surgery*, vol. 253, no. 3, pp. 453–469, 2011.

[12] A. Bujold and L. A. Dawson, "Stereotactic radiation therapy and selective internal radiation therapy for hepatocellular carcinoma," *Cancer/Radiothérapie*, vol. 15, no. 1, pp. 54–63, 2011.

[13] C. Ritter, M. E. Andrades, A. Reinke, S. Menna-Barreto, J. C. F. Moreira, and F. Dal-Pizzol, "Treatment with *N*-acetylcysteine plus deferoxamine protects rats against oxidative stress and improves survival in sepsis," *Critical Care Medicine*, vol. 32, no. 2, pp. 342–349, 2004.

[14] B. Raynard, A. Balian, D. Fallik et al., "Risk factors of fibrosis in alcohol-induced liver disease," *Hepatology*, vol. 35, no. 3, pp. 635–638, 2002.

[15] P. Mandrekar, S. Bala, D. Catalano, K. Kodys, and G. Szabo, "The opposite effects of acute and chronic alcohol on lipopolysaccharide-induced inflammation are linked to IRAK-M in human monocytes," *The Journal of Immunology*, vol. 183, no. 2, pp. 1320–1327, 2009.

[16] H. Zhou, M. Yu, K. Fukuda et al., "IRAK-M mediates Toll-like receptor/IL-1R-induced NFκB activation and cytokine production," *The EMBO Journal*, vol. 32, no. 4, pp. 583–596, 2013.

[17] B. Venkatesan, A. J. Valente, S. D. Prabhu, P. Shanmugam, P. Delafontaine, and B. Chandrasekar, "EMMPRIN activates multiple transcription factors in cardiomyocytes, and induces interleukin-18 expression via Rac1-dependent PI3K/Akt/IKK/NF-κB and MKK7/JNK/AP-1 signaling," *Journal of Molecular and Cellular Cardiology*, vol. 49, no. 4, pp. 655–663, 2010.

[18] R. G. Baker, M. S. Hayden, and S. Ghosh, "NF-κB, inflammation, and metabolic disease," *Cell Metabolism*, vol. 13, no. 1, pp. 11–22, 2011.

[19] W. Zhong, K. Qian, J. Xiong, K. Ma, A. Wang, and Y. Zou, "Curcumin alleviates lipopolysaccharide induced sepsis and liver failure by suppression of oxidative stress-related inflammation via PI3K/AKT and NF-κB related signaling," *Biomedicine & Pharmacotherapy*, vol. 83, pp. 302–313, 2016.

[20] C. Dan, B. Jinjun, H. Zi-Chun et al., "Modulation of TNF-α mRNA stability by human antigen R and miR181s in sepsis-induced immunoparalysis," *EMBO Molecular Medicine*, vol. 7, no. 2, pp. 140–157, 2015.

[21] M. Papurica, A. F. Rogobete, D. Sandesc et al., "The expression of nuclear transcription factor kappa B (NF-κB) in the case of critically Ill polytrauma patients with sepsis and its interactions with microRNAs," *Biochemical Genetics*, vol. 54, no. 4, pp. 337–347, 2016.

[22] Y. Zhang, S. Wang, Y. Li, Z. Xiao, Z. Hu, and J. Zhang, "Sophocarpine and matrine inhibit the production of TNF-α and IL-6 in murine macrophages and prevent cachexia-related symptoms induced by colon26 adenocarcinoma in mice," *International Immunopharmacology*, vol. 8, no. 13-14, pp. 1767–1772, 2008.

[23] Y. Gao, G. Li, C. Li et al., "Anti-nociceptive and anti-inflammatory activity of sophocarpine," *Journal of Ethnopharmacology*, vol. 125, no. 2, pp. 324–329, 2009.

[24] B. Guo, C. Li, Z. Deng et al., "A new method for measurement of (−)-sophocarpine, a candidate therapeutic for viral myocarditis, in plasma: application to a toxicokinetic study in beagle dogs," *Rapid Communications in Mass Spectrometry*, vol. 19, no. 19, pp. 2840–2848, 2005.

[25] Y. Gao, W. Jiang, C. Dong et al., "Anti-inflammatory effects of sophocarpine in LPS-induced RAW 264.7 cells via NF-κB and MAPKs signaling pathways," *Toxicology In Vitro*, vol. 26, no. 1, pp. 1–6, 2012.

[26] E. Oztürk, S. Demirbilek, A. Köroğlu et al., "Propofol and erythropoietin antioxidant properties in rat brain injured tissue," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 32, no. 1, pp. 81–86, 2008.

[27] S. Guo, Y. Zhang, Z. Wang, Y. Yu, and G. Wang, "Intraperitoneal gardiquimod protects against hepatotoxicity through inhibition of oxidative stress and inflammation in mice with sepsis," *Journal of Biochemical and Molecular Toxicology*, vol. 31, no. 8, article e21923, 2017.

[28] H. Jaeschke, "Reactive oxygen and mechanisms of inflammatory liver injury: present concepts," *Journal of Gastroenterology and Hepatology*, vol. 26, pp. 173–179, 2011.

[29] C. M. Raval, J. L. Zhong, S. A. Mitchell, and R. M. Tyrrell, "The role of Bach1 in ultraviolet A-mediated human heme oxygenase 1 regulation in human skin fibroblasts," *Free Radical Biology & Medicine*, vol. 52, no. 1, pp. 227–236, 2012.

[30] R. Franco and J. A. Cidlowski, "Glutathione efflux and cell death," *Antioxidants & Redox Signaling*, vol. 17, no. 12, pp. 1694–1713, 2012.

[31] V. Sanchez-Valle, N. C. Chavez-Tapia, M. Uribe, and N. Mendez-Sanchez, "Role of oxidative stress and molecular changes in liver fibrosis: a review," *Current Medicinal Chemistry*, vol. 19, no. 28, pp. 4850–4860, 2012.

[32] J. K. Kiecolt-Glaser, K. J. Preacher, R. C. MacCallum, C. Atkinson, W. B. Malarkey, and R. Glaser, "Chronic stress and age-related increases in the proinflammatory cytokine IL-6," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 15, pp. 9090–9095, 2003.

[33] I. B. Slimen, T. Najar, A. Ghram, H. Dabbebi, M. Ben Mrad, and M. Abdrabbah, "Reactive oxygen species, heat stress and oxidative-induced mitochondrial damage. A review," *International Journal of Hyperthermia*, vol. 30, no. 7, pp. 513–523, 2014.

[34] Y. Funato, T. Michiue, M. Asashima, and H. Miki, "The thioredoxin-related redox-regulating protein nucleoredoxin inhibits Wnt-β-catenin signalling through dishevelled," *Nature Cell Biology*, vol. 8, no. 5, pp. 501–508, 2006.

[35] P. H. Zapelini, G. T. Rezin, M. R. Cardoso et al., "Antioxidant treatment reverses mitochondrial dysfunction in a sepsis animal model," *Mitochondrion*, vol. 8, no. 3, pp. 211–218, 2008.

[36] Y. Ren, X. Yang, X. Niu, S. Liu, and G. Ren, "Chemical characterization of the avenanthramide-rich extract from oat and its effect on D-galactose-induced oxidative stress in mice," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 1, pp. 206–211, 2011.

[37] S. Azarabadi, H. Abdollahi, M. Torabi, Z. Salehi, and J. Nasiri, "ROS generation, oxidative burst and dynamic expression profiles of ROS-scavenging enzymes of superoxide dismutase (*SOD*), catalase (*CAT*) and ascorbate peroxidase (*APX*) in

response to *Erwinia amylovora* in pear (*Pyrus communis* L)," *European Journal of Plant Pathology*, vol. 147, no. 2, pp. 279–294, 2017.

[38] L. Chen, S. Li, X. Guo, P. Xie, and J. Chen, "The role of GSH in microcystin-induced apoptosis in rat liver: involvement of oxidative stress and NF-κB," *Environmental Toxicology*, vol. 31, no. 5, pp. 552–560, 2014.

[39] Q. H. Huang, L. Q. Xu, Y. H. Liu et al., "Polydatin protects rat liver against ethanol-induced injury: involvement of CYP2E1/ROS/Nrf2 and TLR4/NF-κB p65 pathway," *Evidence-Based Complementary and Alternative Medicine*, vol. 2017, Article ID 7953850, 14 pages, 2017.

[40] R. H. Shih, C. Y. Wang, and C. M. Yang, "NF-kappaB signaling pathways in neurological inflammation: a mini review," *Frontiers in Molecular Neuroscience*, vol. 8, p. 77, 2015.

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Research Article

Reduced NLRP3 Gene Expression Limits the IL-1β Cleavage via Inflammasome in Monocytes from Severely Injured Trauma Patients

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Objective. Traumatic injury or severe surgery leads to a profound immune response with a diminished functionality of monocytes and subsequently their IL-1β release. IL-1β plays an important role in host immunity and protection against infections. Its biological activation via IL-1β-precursor processing requires the transcription of inflammasome components and their activation. Deregulated activity of NOD-like receptor inflammasomes (NLR) like NLRP3 that leads to the maturation of IL-1β has been described in various diseases. While the role of other inflammasomes has been studied in monocytes, nothing is known about NLRP3 inflammasome after a traumatic injury. Here, the role of the NLRP3 inflammasome in impaired monocyte functionality after a traumatic injury was analyzed. *Measurements and Main Results*. Ex vivo-*in vitro* stimulation of isolated $CD14^+$ monocytes with lipopolysaccharide (LPS) showed a significantly higher IL-1β secretion in healthy volunteers (HV) compared to trauma patients (TP) after admission. Reduced IL-1β secretion was paralleled by significantly lowered gene expression of NLRP3 in monocytes from TP compared to those of HV. Transfection of monocytes with NLRP3-encoding plasmid recovered the functionality of monocytes from TP regarding the IL-1β secretion. *Conclusions*. This study demonstrates that $CD14^+$ monocytes from TP are significantly diminished in their function and that the presence of NLRP3 components is necessary in recovering the ability of monocytes to produce active IL-1β. This recovery of the NLRP3 inflammasome in monocytes may imply a new target for treatment and therapy of immune suppression after severe injury.

1. Introduction

Each year millions of people sustain injuries with the need for medical treatment, and around five million people died from them in 2013 [1]. Regarding severe traumatic injuries, a biphasic pattern of mortality is evident, as those patients who survive the initial injury are still at risk of death caused by immunological alterations ending in, for example, multiple organ failure in the later clinical course [2, 3]. Despite extensive research to address the immune response after trauma and as observed in intensive care units, the pathophysiology of immunological alterations is not yet fully understood, and the mortality remains high [4]. Experimental data links dysfunction of circulating monocytes to poor outcomes in patients with severe tissue injuries caused by trauma as well [5–7]. The biological immune response to tissue damage implies cytokine secretion which is triggered by either pathogen-associated molecular patterns (PAMP) like lipopolysaccharide (LPS) or damage-associated molecular patterns (DAMP) such as high mobility group box B1 (HMGB1) protein. In trauma, however, functional depression of monocytes leads to a suppressed production of interleukin- (IL-) 1β [6, 8]. On the other hand, lack of IL-1β is associated with immune suppression in critical illness and enhanced susceptibility to lethal infections as shown in mice IL-1 knockout models [9].

IL-1β undergoes a thoroughly controlled process of transcription, translation, and activation. Major sources of IL-1β

include blood monocytes, macrophages, and dendritic cells among others [10, 11]. IL-1β messenger ribonucleic acid (mRNA) transcription is induced upon activation of Toll-like receptors (TLRs) by, for example, LPS or endogenous cytokines [12]. Additional stimuli like adenosine triphosphate (ATP), viral RNA, or pore-forming toxins activate the intracellular NOD-like receptor sensor molecule containing pyrin domain (NLRP) inflammasome and thereby the IL-1β converting enzyme caspase-1 to cleave IL-1β-precursor into its active form [13, 14]. As multiprotein complexes, inflammasomes consist of the effector protein caspase-1, an apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD) (ASC) and a sensor protein from the NOD-like receptor (NLR family) like NLRP1, NLRP3, or absent-in-melanoma (AIM)2 among others [15]. The presence of each inflammasome component is imperative for its formation and activation [15]. Unlike other inflammasomes, NLRP3 is triggered by a broader range of stimuli like uric acid crystals or asbestos and may, therefore, be more fundamental for the host defense [16, 17].

Impaired functionality of monocytes occurs early after trauma upon the admission of patients to the emergency departments as well as during the later clinical course [18, 19]. In monocytes isolated from patients with septic shock, the lack of NLRP1 mRNA correlated with the impairment of monocytes [20]. Our group showed that functional depression of monocyte from trauma patients went along with reduced NLRP1 mRNA expression and moreover that restoring NLRP1 via transfection recovered the functional activity of monocytes [19]. In this study, we examined if the NLRP3 inflammasome in monocytes isolated from trauma patients plays a mechanistically important role in reduced IL-1β secretion as well. To our knowledge, this has not been done before and could lead to the identification of crucial therapeutical targets against infections and organ failure in patients after severe trauma.

2. Patients and Methods

2.1. Ethics. This study was performed in the University Hospital Frankfurt of Goethe University. The study was approved by the institutional ethics committee and was performed in accordance with the Declaration of Helsinki and following STROBE guidelines [21]. In accordance with ethical standards, all enrolled subjects, healthy volunteers as well as patients, signed the written informed consent form by themselves or the written informed consent was obtained from the legally authorized representative.

2.2. Patients. Patients between 18 and 80 years of age with an Injury Severity Score (ISS) ≥ 16 and a history of acute blunt or penetrating trauma were included. Based on the Abbreviated Injury Scale (AIS) of 2008, the ISS was calculated [22, 23]. Exclusion criteria consisted of preexisting immunological disorders, immune-suppressive and anticoagulant medication, acute myocardial infarction, thromboembolic events, and/or lethal injury. Vital signs were measured upon arrival at the emergency department (ED), and blood samples were also obtained on admission to ED. Twenty patients with traumatic injury (trauma patients (TP)) and 10 healthy volunteers (HV) for comparison were included.

2.3. Blood Sampling. The blood samples were acquired immediately after the patient's admission to ED using ethylenediaminetetraacetic acid (EDTA) monovettes (Sarstedt, Nürmbrecht, Germany) and kept at room temperature due to subsequent functional assays.

2.4. Isolation of $CD14^+$ Monocytes. Fresh blood samples from TP and HV were used for $CD14^+$ monocyte isolation. Briefly, monocytes were isolated using Ficoll's density gradient ($d = 1.077$ g/mL, Biochrom AG, Berlin, Germany) and centrifugation at 600g for 20 min at room temperature [19]. Subsequently, the mononuclear cell layer was removed and washed in MACS buffer (2 mM EDTA, 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS, w/o Mg^{2+} and Ca^{2+})). The washing procedure was repeated twice. Then, by applying positive selection, anti-CD14-coated magnetic beads were used according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Flow cytometric analysis verified the purity of the isolated $CD14^+$ cells (>97%). After isolation and purity determination, 1×10^5 $CD14^+$ monocytes/200 μL RPMI-1640 (Seromed, Berlin, Germany; 48-well multidish plates, BD Bioscience, Franklin Lakes, NJ, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco, Karlsruhe, Germany) and 20 mM HEPES buffer (Sigma, Deisenhofen, Germany) were cultured for 1 h at 37°C under 5% CO_2. Then, nonadherent cells were removed, and adherent $CD14^+$ monocytes were washed again and cultured in 200 μL RPMI-1640 with supplements as described above and before [19].

2.5. Ex Vivo-In Vitro Stimulation with LPS and IL-1β Release. $CD14^+$ isolated monocytes were stimulated with LPS (10 μg/mL, *E. coli* 0127: B8, Sigma) in 200 μL RPMI-1640 with supplements per well and cultured for 24 h at 37°C and 5% CO_2 as described previously [19]. The supernatant was removed and stored at −80°C after 24 hours. The samples were used for an IL-1β ELISA assay (Quantikine®, Human IL-1β/IL-1F2 Immunoassay ELISA, R&D Systems) according to the manufacturer's instructions. The adherent cells were used for RNA isolation and gene expression analyses.

2.6. RNA Isolation and Semiquantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). The RNA from cultured $CD14^+$ monocytes was isolated using the RNeasy-system (Qiagen, Hilden, Germany) according to the manufacturer's instructions and as described before [19]. RNase-Free DNase Set was applied to remove any residual amounts of DNA according to the manufacturer's instructions (Qiagen), and both RNA quality and quantity were determined using the NanoDrop ND-1000 device (NanoDrop Technologies, Wilmington, DE, USA). RNA was stored at −80°C until cDNA synthesis and qRT-PCR as described previously [24]. Briefly, 100 ng of total RNA was reversely transcribed using the Affinity Script QPCR-cDNA

Synthesis Kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. Gene-specific primers for human NLRP3 (NM_183395, UniGene number: Hs.159483, Cat. number: PPH13170A) and GAPDH (NM 002046, UniGene number: Hs.592355, Cat. number: PPH00150E) as reference gene were purchased from SABiosciences (SuperArray, Frederick, MD, USA). The PCR reaction was set up with 1x RT [2] SYBR Green/Rox qPCR Master Mix (SABiosciences) in a 25 μL volume and a two-step amplification protocol with initial denaturation at 95°C for 10 min followed by 40 cycles with 15 s denaturation at 95°C, and 60 s annealing/extension at 60°C was applied. The specificity of amplification products was controlled by a melting-curve analysis. And qRT-PCR was carried out on a Stratagene MX3005p QPCR System (Stratagene).

Relative expression of target mRNA in each sample was calculated using the comparative threshold cycle (CT) method ($\Delta\Delta$CT method) [25]. Here, the amount of target mRNA was normalized to the amount of housekeeping GAPDH to provide ΔCT and subsequently to a calibrator consisting of samples obtained from unstimulated control cells (fold change to unstimulated).

2.7. Reconstitution of NLRP3 in Isolated CD14$^+$ Monocytes. Isolated CD14$^+$ monocytes were transfected with 0.6 μg/mL of either human NLRP3 (HS_NLRP3_IM_1, gene ID: 114548, NM_001079821, EIM0044989) or the positive/plasmid control (PC, $n = 6$) CDC2 (delivered with the kit) expression plasmids (vector backbone pQE-TriSystem-6, QIAgenes Expression Kit Insect/Mammalia, Qiagen, Hilden, Germany) in the FCS-free culture medium. And the transfection reagent Attractene (5:1 DNA, Qiagen) was used. The medium was substituted with RPMI-1640 culture medium supplemented as described above two hours later. 48 hours later, cells were challenged with LPS (10 μg/mL, *E. coli* 0127: B8, Sigma) and incubated for additional 24 hours before cell extracts for gene and protein as well as supernatants for IL-1β analyses were obtained. The viability of isolated cells gradually decreased over time; however, staining with propidium iodide (PI, Sigma Aldrich Ltd.) and subsequent microscopical evaluation have shown that there was a minimal cell death upon transfection. Monocytes that regularly displayed at least 75% viability were applied for the experiments.

2.8. Verification of the Transfection via Immunohistological Staining. Isolated monocytes from healthy volunteers were used to monitor the transfection efficiency. In short, 48 hours after transfection, isolated cells were washed (PBS, w/o Mg^{2+} and Ca^{2+}) and fixed using 100 μL solution A (FIX & PERM® Cell Fixation & Permeabilization Kit, ADG Bioresearch GmbH, Kaumberg, Austria) for 10 min. Then, cells were washed again and incubated with a mouse monoclonal Penta-His antibody directed against His-tagged recombinant protein (Qiagen) and resuspended in 100 μL solution B (from the same kit) for 15 min at room temperature. After another washing procedure with 0.5% BSA in PBS, cells were stained with a secondary NL557 antimouse antibody (R&D Systems, Minneapolis, USA) for 30 min. After a final PBS washing step, cells were microscopically analyzed using the Axio Observer Z1 (Zeiss, Göttingen, Germany).

2.9. Protein Isolation and Western Blotting for NLRP3 Components. After the transfection process, the cells were lysed in the lysing buffer (50 mM Tris base-hydrochloric acid (Tris-HCl), pH 7.4; 150 mM sodium chloride (NaCl); 1% tergitol-type NP-40; and 0.25% Na-deoxycholate) containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF); 1 mM ethylenediaminetetraacetic acid (EDTA); aprotinin, pepstatin, and leupeptin (1 μg/mL each); 1 mM sodium orthovanadate (Na_3VO_4); and 1 mM sodium fluoride (NaF)) at 4°C. Then, a centrifugation for 30 min at 4°C at 20.000 ×g followed. Supernatants were stored at −80°C for subsequent western blotting analysis.

Electrophoresis on 12% polyacrylamide SDS gels was used to separate lysates (50 μg protein) which were then transferred to Hybond-C Extra supported nitrocellulose membranes (Amersham-Buchler, Braunschweig, Germany). NLRP3 was detected using mouse monoclonal NLRP3/NALP3 (human), mAb (Nalpy3-a) antibody (Enzo Life Sciences, NY, USA), and His by using mouse monoclonal His-probe antibody (H-3) (all Santa Cruz Biotechnology, Santa Cruz, CA, USA). β-actin as internal loading control was detected by a mouse anti-β-actin antibody (Sigma, Taufkirchen, Germany). Blots were blocked (10% nonfat dry milk in 1 mM Tris, 150 mM NaCl, pH 7.4) for 1 hour, then incubated for 1 hour at RT with primary antibody (diluted according to manufacturer's instructions in blocking buffer with 0.5% Tween 20 and 0.5% BSA), and then incubated for another hour with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) diluted 1 : 1000 in blocking buffer with 0.5% Tween 20 and 0.5% bovine serum albumin at RT as described before [19]. ECL™ western blot detection reagents (GE Healthcare, Munich, Germany) were used for protein detection. The signals were digitized using the FUSION-FX7 machine and Bio-1D software (Peqlab Biotechnologies, Erlangen, Germany).

2.10. Statistical Analysis. Kolmogorov-Smirnov test was used to verify the normality of data. Student's *t*-test with Welch correction and one-way analysis of variance (ANOVA) with Dunn post hoc test were applied for comparison among different groups. A p value below 0.05 was considered statistically significant. Data are given as mean ± standard error of the mean (SEM). And statistical analysis was performed with GraphPad Prism 6.0 software (GraphPad Software Inc. San Diego, CA).

3. Results

3.1. Study Subjects Represent Severely Injured Trauma Patients. In this study, 20 patients with traumatic injury as well as 10 healthy volunteers were enrolled. Most of the patients were male (15 TP, 75% versus 7 HV, 70%, Table 1). The mean age of study subjects was 38.95 ± 3.50 years for TP and marginally younger in HV with 35.80 ± 3.99 years (Table 1). The enrolled cohort of patients had severe injuries as the mean ISS was 30.5 ± 1.80 points. The stay at the

Table 1: Patient's characteristics. ctrl: healthy volunteer controls; d: days; ICU: intensive care unit; ISS: Injury Severity Score; n.s.: not significant; TP: trauma patients; vs.: versus; y: years.

Characteristics	TP ($n = 20$)	ctrl ($n = 10$)	p value (TP versus ctrl)
Age (y)	38.95 ± 3.50	35.80 ± 3.99	n.s.
Sex (male, n)	15	7	n.s.
ISS	30.5 ± 1.80	—	
ICU stay (d)	12.90 ± 1.50	—	
Hospital stay (d)	20.95 ± 2.99	—	

intensive care unit (ICU) was 12.90 ± 1.50 days. The mean hospital duration was 20.95 ± 2.99 days (Table 1). This cohort of severely injured trauma patients is comparable to preceding studies and represents this cohort of patients with severe and major injury as often observed in the ICU [26–28].

3.2. Lowered LPS Response in Isolated CD14$^+$ Monocytes Is Paralleled by Decreased NLRP3 Gene Expression after Trauma. To determine that isolated monocytes react as described before in own studies and in literature, the IL-1β secretion capabilities after LPS challenge were measured using an ELISA assay [19, 20]. The ex vivo-*in vitro* LPS-stimulated IL-1β secretion was significantly enhanced in HV and TP after LPS challenge compared to the corresponding nonstimulated cells ($p < 0.05$, Figure 1(a)). Compared to the IL-1β response of HV isolated, CD14$^+$ monocytes from TP released significantly less IL-1β upon LPS stimulation ($p < 0.05$, Figure 1(a)). Therefore, the isolated CD14$^+$ monocytes of our cohort display the previously described impaired function of these cells after trauma.

To verify that the diminished function of CD14$^+$ monocytes after LPS challenge in TP is associated with NLRP3, NLRP3 gene expression was measured. Gene expression of NLRP3 in monocytes from HV was significantly increased compared to that in unstimulated cells ($p < 0.05$, Figure 1(b)). In isolated CD14$^+$ monocytes from TP, LPS stimulation did not lead to a significantly increased NLRP3 gene expression, although there was a trend to an increase compared to nonstimulated controls (Figure 1(b)). When comparing both LPS-challenged monocytes from HV and TP, NLRP3 gene expression was significantly decreased in CD14$^+$ monocytes from TP versus HV ($p < 0.05$, Figure 1(b)). This data underlines diminished immune regulatory capabilities of the investigated monocytes from patients with severe injury and directly indicates an association between reduced IL-1β response after LPS challenge and NLRP3 gene expression.

3.3. NLRP3 Transfection of Isolated CD14$^+$ Monocytes. Isolated CD14$^+$ cells were transfected with either plasmid coding for NLRP3 or plasmid control (PC) to understand the role of NLRP3 inflammasome elements for monocyte functionality. In Figure 2(a), positive red staining of the His-tag in isolated monocytes verifies the expression of encoded genes in plasmid by recognizing the His-tag fused to control vector. The left panel of immunostaining displays no positive tag staining in nontransfected cells, while the middle panel shows positive staining of PC and indicates, therefore, an expression of the PC plasmid (Figure 2(a)). In the right panel, the NLRP3 expression is confirmed via positive red staining (Figure 2(a)).

Protein expression of NLRP3 was verified by western blotting of whole-cell protein extracts of isolated CD14$^+$ monocytes after NLRP3 transfection and LPS stimulation or of ctrl (Figure 2(b)). Due to the limited availability of biomaterial from TP at admission to ED, this assay was performed only for monocytes of HV. A His-tag antibody was utilized to identify positive bands with a similar size as of those that were detected with NLRP3 antibody after transfection. Successful transfection of CD14$^+$ cells from HV with NLRP3 is confirmed by this result (Figure 2(b)).

To verify the transfection efficiency, NLRP3 gene expression was determined after transfection in CD14$^+$ cells from TP as well as HV (Figure 2(c)). Transfection with NLRP3 led to a significant rise of NLRP3 gene expression in TP and HV when compared to nontransfected or PC-transfected controls after LPS stimulation ($p < 0.05$, Figure 2(c)). Transfection with the PC was not significantly distinctive in its NLRP3 gene expression compared to control monocytes after LPS stimulation (Figure 2(c)). Transfection with NLRP3 in isolated monocytes from TP at ED admission did not reach gene expression levels of NLRP3 as compared to HV upon NLRP3 transfection, but the difference was not significant between those two groups (Figure 2(c)).

3.4. IL-1β Release after LPS Stimulation Is Recovered after NLRP3 Transfection of CD14$^+$ Monocytes. To investigate how isolated monocytes from TP transfected with NLRP3-encoding plasmid will perform in regard to functionality, CD14$^+$ cells were treated with LPS after transfection, and IL-1β secretion was measured. This was also implemented for monocytes from HV as control. The functionality of non-transfected cells with regard to IL-1β release was significantly decreased in ctrl monocytes from TP compared to ctrl monocytes from HV ($p < 0.05$, Figure 3). In NLRP3-transfected cells from HV, the IL-1β secretion after LPS stimulation was significantly higher compared to nontransfected monocytes from HV or TP, respectively ($p < 0.05$, Figure 3). The transfection of isolated CD14$^+$ cells from TP with NLRP3 gene-encoding plasmid significantly enhanced IL-1β release after LPS challenge compared to that of nontransfected cells from TP ($p < 0.05$, Figure 3). This data indicates that NLRP3 transfection of functionally depressed monocytes after trauma enhances their IL-1β secretory capability.

4. Discussion

Monocytes constitute an integral part of the innate immunity defense as they early detect PAMP or DAMP via their pattern recognition receptors (PRR). The intracellular family of NOD-like receptors (NLR) as PRR is essential in activating host response to pathogens [29]. A prominent member of the NLR family is the receptor NLRP3 which is part of the NLRP3 inflammasome, an intracellular multiprotein complex that cleaves inactive IL-1β precursor to its active form

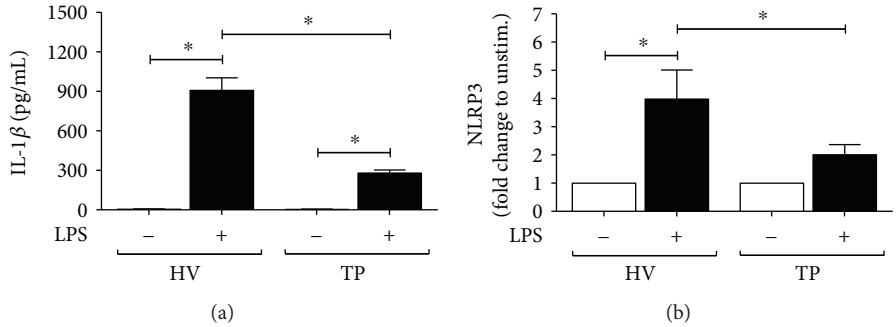

Figure 1: Trauma reduces IL-1β secretion and NLRP3 expression in isolated CD14$^+$ monocytes. (a) Isolated CD14$^+$ cells from peripheral mononuclear blood obtained either from trauma patients (TP, $n = 20$) on admission or healthy volunteers (HV, $n = 10$) were incubated and treated as described in Patients and Methods. IL-1β secretion (pg/mL) after stimulation with lipopolysaccharide (LPS) was determined by ELISA. (b) Gene expression analysis of NLRP3 as fold change to unstimulated CD14$^+$ monocytes which were isolated and treated with LPS as described. Data are shown as mean ± SEM. $^*p < 0.05$ *versus* indicated.

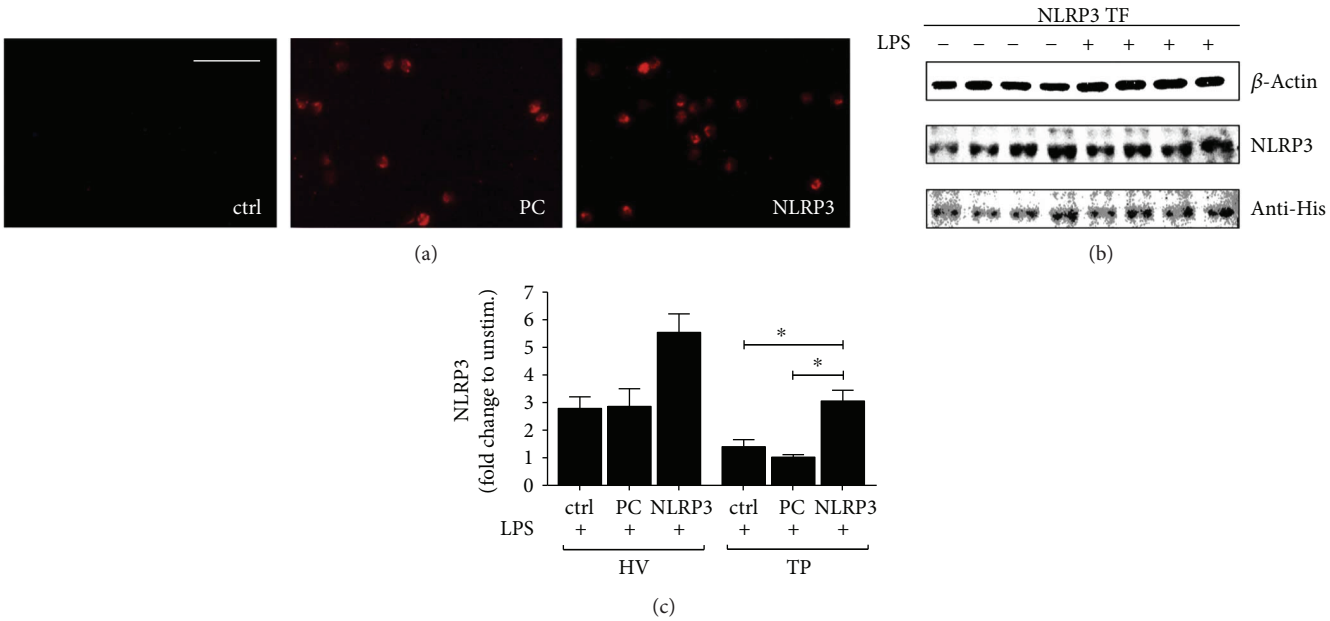

Figure 2: Transfection of isolated CD14$^+$ cells with the NLRP3 plasmid increases NLRP3 gene and protein expression. (a) Immunohistological staining of human CD14$^+$ monocytes isolated from healthy volunteers (HV). Left panel presents nontransfected cells (ctrl), middle panel displays CD14$^+$ cells transfected with plasmid control (PC), while the right panel shows cells transfected with the NLRP3 plasmid. Representative photos are shown. (b) Isolated CD14$^+$ monocytes were transfected with NLRP3 plasmid and either untreated or treated with lipopolysaccharide (LPS). Protein extracts were isolated from the cells and analyzed via western blotting as described in Patients and Methods to detect indicated proteins. Representative blots of four independent experiments are shown. (c) Isolated CD14$^+$ monocytes from either HV or TP were transfected with NLRP3-encoding plasmid (NLRP3) or plasmid control (PC) or remained untreated (ctrl). Cells were then stimulated with LPS and gene analysis of NLRP3 was represented. Data are shown as mean ± SEM. $^*p < 0.05$ *versus* indicated.

via caspase-1 [17]. The cytokine secretion by monocytes is also required for the activation of adaptive immunity through T cells [29]. Severe injury or surgery can lead to compromised host defense ensuing sepsis and multiple organ failure [30, 31]. Here, it became evident that impaired capability of monocytes to secrete cytokines predicted high mortality caused by (infectious) complication after severe trauma [8, 18, 32]. Therefore, the inflammasome complexes became a major focus of research in recent years. With regard to trauma, NLRP1 has been studied by several groups. For instance, Fahy et al. described that low levels of mRNA for NLRP1 and caspase-1 in monocyte ex vivo were linked to sepsis and survival in septic shock [20]. Our group described a diminished functionality of monocytes in severely injured trauma patients and uncovered in ex vivo-*in vitro* analyses that reduced NLRP1 gene expression was a contributing factor to monocyte suppression after trauma [19]. The present study reveals that the NLRP3 inflammasome is apparently affected by trauma as well and diminished in its function due to decreased NLRP3 gene expression after trauma (Figure 1(a)). Furthermore, downregulated NLRP3 gene expression was paralleled by a decreased IL-1β response after

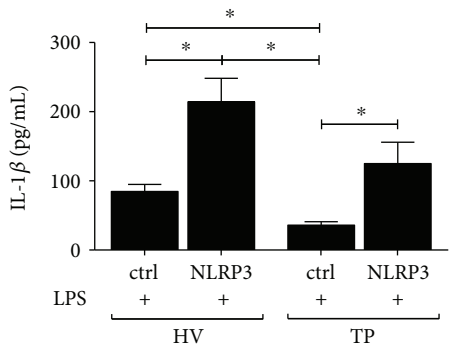

Figure 3: Transfecting $CD14^+$ monocytes with NLRP3 recovers their functionality. $CD14^+$ monocytes were isolated either from healthy volunteers (HV) or from trauma patients (TP) after the admission. Subsequently, cells were transfected with the NLRP3 expression plasmid or with plasmid control (ctrl) before stimulation with lipopolysaccharide (LPS). In the supernatants, secretion of IL-1β was determined by ELISA. Data are shown as mean ± SEM. $^*p < 0.05$ *versus* indicated.

LPS challenge. As described by others before, NLRP3 inflammasome is essential in the synthesis of mature IL-1β, which can be used ex vivo to asses NLRP3 activity in human monocyte-derived cells [10]. However, there is data conflicting our findings. A Brazilian group reported increased ex vivo NLRP3 gene expression in patients with septic shock, while NLRP1 and NOD1 were decreased [33]. However, they used peripheral blood mononuclear cells that include lymphocytes and NK cells as well, a difference that may explain the divergence to our data [33].

In the 1960s, Muckle and Wells first described a disease with transient attacks of fever, urticaria, and deafness in a family in Derbyshire, England [34]. Hoffman later described a related disease called "Familial Autoinflammatory Cold Syndrome" that became symptomatic when patients were exposed to cold temperatures [35]. The underlying genetic mutation coded for a protein that was termed cryopyrin and later NLRP3 [35]. Since then, NLRP3 mutation and hyperactivation have been linked to gout, diabetes type 2, familial Mediterranean fever, and rheumatic disease among many others [36]. However, in terms of traumatic injury, the activation of NLRP3 is essential for tissue repair via the so-called "regenerative inflammation" [37]. Furthermore, NLRP3 knockout mice models display a compromised host defense against *Candida*, *Toxoplasma gondii*, or *Salmonella* among others [38–40]. Despite all this research, the modulation of NLRP3 gene expression in traumatic injury has been scarcely studied. Effective assembly of the NLRP3 inflammasome complex and subsequent release of IL-1β requires the components to be fully available intracellularly [15]. Whole-blood assays by Singh et al. revealed enhanced NLRP3 mRNA levels in patients with septic shock that correlated with severity of the illness [41]. Nonetheless, the contribution of other cells than monocytes to the NLRP3 mRNA levels has to be taken into account, and additionally, the study population was rather small ($n = 29$) [41]. Another interesting work by a German group investigated inflammasome modulation in human monocytes after cardiopulmonal resuscitation (CPR) [42]. They presented data of elevated NLRP3 mRNA levels in monocytes 24 hours after CPR, while NLRP1 levels were diminished [42]. This correlates with further above-described findings of Fahy et al. Yet, when comparing the 30-day survival, those patients that had lower levels of NLRP3 mRNA in monocytes 48 hours after CPR had significantly lower survival in the 30-day period [42]. This provides insight into a protective role of NLRP3 in critically ill patients. The mode of injury (cardiac arrest and CPR versus severe traumatic injury) may explain our findings of lower NLRP3 mRNA levels in TP after admission. It is also worth noting that Asmussen et al. measured mRNA levels only and did not study the cells further.

In this study, we show that a reconstitution of the lacking NLRP3 in human monocytes from trauma patients can recover the NLRP3 gene expression and, apparently, thereby provide the function to secrete active IL-1β (Figures 2(a)–2(c) and 3). This is a novel finding that to our knowledge has not been reported before. Similar findings have been shown by our group for NLRP1 inflammasome [19].

However, there are limitations that have to be considered. First, our study population was rather small ($n = 20$), and further protein analyses were not possible due to limited biomaterial of trauma patients. Secondly, transfection of $CD14^+$ monocytes from healthy volunteers led to an increased NLRP3 activity after LPS challenge just as well as in monocytes from trauma patients. Recent research of the aforementioned NLRP3 mutation syndromes may provide an explanation. It has been shown that patients with NLRP3 mutation display a maximally increased IL-1β secretion after LPS challenge [43].

While our group investigated NLRP1 before and NLRP3 inflammasome in this study, further research in this field may provide new perspectives in the treatment of critically ill patients. Larger study population and subpopulation analyses of septic and septic shock patients may reveal a certain pathology that may benefit from enhanced inflammasome activity. It also remains to be evaluated in preclinical *in vivo* models if an ex vivo transfection of monocytes and their subsequent retransfusion will lead to improved outcomes. In summary, associative data from this study indicate that the contribution of NLRP3 inflammasome to host defense after trauma is apparently larger than previously anticipated.

Abbreviations

AIS: Abbreviated Injury Scale
ASC: Apoptosis-associated speck-like protein containing CARD
BSA: Bovine serum albumin
CARD: Caspase activation and recruitment domain
CASP1: Caspase-1
CD: Cluster of differentiation
ctrl: Control
DAMP: Damage-associated molecular pattern
ED: Emergency department
ELISA: Enzyme-linked immunosorbent assay
EDTA: Ethylenediaminetetraacetic acid

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
HV: Healthy volunteers
ICU: Intensive care unit
IL: Interleukin
ISS: Injury Severity Score
LRR: Leucine-rich repeats
LPS: Lipopolysaccharide
mRNA: Messenger ribonucleic acid
NLR: NOD-like receptor
NLRP: NLR sensor molecule containing pyrin domain
PBS: Phosphate-buffered saline
PAMP: Pathogen-associated molecular pattern
PC: Plasmid control
PRR: Pattern recognition receptor
PYCARD: Gene encoding for ASC
TP: Trauma patients.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no competing interests.

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References

[1] J. A. Haagsma, N. Graetz, I. Bolliger et al., "The global burden of injury: incidence, mortality, disability-adjusted life years and time trends from the Global Burden of Disease study 2013," *Injury Prevention*, vol. 22, no. 1, pp. 3–18, 2016.

[2] V. M. Stoecklein, A. Osuka, and J. A. Lederer, "Trauma equals danger–damage control by the immune system," *Journal of Leukocyte Biology*, vol. 92, no. 3, pp. 539–551, 2012.

[3] T. Tsukamoto, R. S. Chanthaphavong, and H. C. Pape, "Current theories on the pathophysiology of multiple organ failure after trauma," *Injury*, vol. 41, no. 1, pp. 21–26, 2010.

[4] L. Hutchings, P. Watkinson, J. D. Young, and K. Willett, "Defining multiple organ failures after major trauma: a comparison of the Denver, Sequential Organ Failure Assessment, and Marshall scoring systems," *Journal of Trauma and Acute Care Surgery*, vol. 82, no. 3, pp. 534–541, 2017.

[5] V. Faivre, A. C. Lukaszewicz, and D. Payen, "Downregulation of blood monocyte HLA-DR in ICU patients is also present in bone marrow cells," *PLoS One*, vol. 11, no. 11, article e0164489, 2016.

[6] C. Kirchhoff, P. Biberthaler, W. E. Mutschler, E. Faist, M. Jochum, and S. Zedler, "Early down-regulation of the pro-inflammatory potential of monocytes is correlated to organ dysfunction in patients after severe multiple injury: a cohort study," *Critical Care*, vol. 13, no. 3, p. R88, 2009.

[7] K. Muthu, L. K. He, K. Melstrom, A. Szilagyi, R. L. Gamelli, and R. Shankar, "Perturbed bone marrow monocyte development following burn injury, and sepsis promote hyporesponsive monocytes," *Journal of Burn Care & Research*, vol. 29, no. 1, pp. 12–21, 2008.

[8] S. Wutzler, M. Maier, M. Lehnert et al., "Suppression and recovery of LPS-stimulated monocyte activity after trauma is correlated with increasing injury severity: a prospective clinical study," *The Journal of Trauma*, vol. 66, no. 5, pp. 1273–1280, 2009.

[9] C. A. Dinarello, "Immunological and inflammatory functions of the interleukin-1 family," *Annual Review of Immunology*, vol. 27, no. 1, pp. 519–550, 2009.

[10] M. V. Fernandez, E. A. Miller, and N. Bhardwaj, "Activation and measurement of NLRP3 inflammasome activity using IL-1β in human monocyte-derived dendritic cells," *Journal of Visualized Experiments*, no. 87, article e51284, 2014.

[11] E. Hadadi, B. Zhang, K. Baidzajevas et al., "Differential IL-1β secretion by monocyte subsets is regulated by Hsp27 through modulating mRNA stability," *Scientific Reports*, vol. 6, no. 1, article 39035, 2016.

[12] F. G. Bauernfeind, G. Horvath, A. Stutz et al., "Cutting edge: NF-κB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression," *The Journal of Immunology*, vol. 183, no. 2, pp. 787–791, 2009.

[13] L. Franchi, T. Eigenbrod, R. Munoz-Planillo et al., "Cytosolic double-stranded RNA activates the NLRP3 inflammasome via MAVS-induced membrane permeabilization and K+ efflux," *The Journal of Immunology*, vol. 193, no. 8, pp. 4214–4222, 2014.

[14] W. Sha, H. Mitoma, S. Hanabuchi et al., "Human NLRP3 inflammasome senses multiple types of bacterial RNAs," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 45, pp. 16059–16064, 2014.

[15] Y. He, H. Hara, and G. Nunez, "Mechanism and regulation of NLRP3 inflammasome activation," *Trends in Biochemical Sciences*, vol. 41, no. 12, pp. 1012–1021, 2016.

[16] C. Dostert, V. Petrilli, R. Van Bruggen, C. Steele, B. T. Mossman, and J. Tschopp, "Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica," *Science*, vol. 320, no. 5876, pp. 674–677, 2008.

[17] O. Gross, C. J. Thomas, G. Guarda, and J. Tschopp, "The inflammasome: an integrated view," *Immunological Reviews*, vol. 243, no. 1, pp. 136–151, 2011.

[18] D. Heftrig, R. Sturm, E. Oppermann et al., "Impaired surface expression of HLA-DR, TLR2, TLR4, and TLR9 in ex vivo-in vitro stimulated monocytes from severely injured trauma patients," *Mediators of Inflammation*, vol. 2017, Article ID 2608349, 12 pages, 2017.

[19] B. Relja, J. P. Horstmann, K. Kontradowitz et al., "Nlrp1 inflammasome is downregulated in trauma patients," *Journal of Molecular Medicine*, vol. 93, no. 12, pp. 1391–1400, 2015.

[20] R. J. Fahy, M. C. Exline, M. A. Gavrilin et al., "Inflammasome mRNA expression in human monocytes during early septic shock," *American Journal of Respiratory and Critical Care Medicine*, vol. 177, no. 9, pp. 983–988, 2008.

[21] E. von Elm, D. G. Altman, M. Egger et al., "The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational

studies," *International Journal of Surgery*, vol. 12, no. 12, pp. 1495–1499, 2014.

[22] S. P. Baker, B. O'Neill, W. Haddon Jr, and W. B. Long, "The injury severity score: a method for describing patients with multiple injuries and evaluating emergency care," *The Journal of Trauma: Injury, Infection, and Critical Care*, vol. 14, no. 3, pp. 187–196, 1974.

[23] C. S. Palmer, J. Lang, G. Russell et al., "Mapping Abbreviated Injury Scale data from 1990 to 1998 versions: a stepping-stone in the contemporary evaluation of trauma," *Injury*, vol. 44, no. 11, pp. 1437–1442, 2013.

[24] B. Relja, D. Henrich, G. Wetzel et al., "Effects of acute ethanol gavage on intestinal integrity after hemorrhage/resuscitation," *Scandinavian Journal of Gastroenterology*, vol. 48, no. 4, pp. 448–458, 2013.

[25] T. D. Schmittgen and K. J. Livak, "Analyzing real-time PCR data by the comparative CT method," *Nature Protocols*, vol. 3, no. 6, pp. 1101–1108, 2008.

[26] R. Sturm, D. Heftrig, K. Mors et al., "Phagocytizing activity of PMN from severe trauma patients in different post-traumatic phases during the 10-days post-injury course," *Immunobiology*, vol. 222, no. 2, pp. 301–307, 2017.

[27] K. Mors, O. Braun, N. Wagner et al., "Influence of gender on systemic IL-6 levels, complication rates and outcome after major trauma," *Immunobiology*, vol. 221, no. 8, pp. 904–910, 2016.

[28] B. Relja, T. Lustenberger, B. Puttkammer et al., "Thrombin-activatable fibrinolysis inhibitor (TAFI) is enhanced in major trauma patients without infectious complications," *Immunobiology*, vol. 218, no. 4, pp. 470–476, 2013.

[29] N. Galbraith, S. Walker, S. Galandiuk, S. Gardner, and H. C. Polk Jr, "The significance and challenges of monocyte impairment: for the ill patient and the surgeon," *Surgical Infections*, vol. 17, no. 3, pp. 303–312, 2016.

[30] E. Faist, A. E. Baue, H. Dittmer, and G. Heberer, "Multiple organ failure in polytrauma patients," *The Journal of Trauma*, vol. 23, no. 9, pp. 775–787, 1983.

[31] H. C. Polk Jr, C. D. George, S. R. Wellhausen et al., "A systematic study of host defense processes in badly injured patients," *Annals of Surgery*, vol. 204, no. 3, pp. 282–299, 1986.

[32] G. Monneret, A. Lepape, N. Voirin et al., "Persisting low monocyte human leukocyte antigen-DR expression predicts mortality in septic shock," *Intensive Care Medicine*, vol. 32, no. 8, pp. 1175–1183, 2006.

[33] K. F. Esquerdo, N. K. Sharma, M. K. C. Brunialti et al., "Inflammasome gene profile is modulated in septic patients, with a greater magnitude in non-survivors," *Clinical and Experimental Immunology*, vol. 189, no. 2, pp. 232–240, 2017.

[34] T. J. Muckle and M. Wells, "Urticaria, deafness, and amyloidosis: a new heredo-familial syndrome," *QJM: An International Journal of Medicine*, vol. 31, pp. 235–248, 1962.

[35] H. M. Hoffman, J. L. Mueller, D. H. Broide, A. A. Wanderer, and R. D. Kolodner, "Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome," *Nature Genetics*, vol. 29, no. 3, pp. 301–305, 2001.

[36] H. M. Hoffman and A. A. Wanderer, "Inflammasome and IL-1β-mediated disorders," *Current Allergy and Asthma Reports*, vol. 10, no. 4, pp. 229–235, 2010.

[37] M. Karin and H. Clevers, "Reparative inflammation takes charge of tissue regeneration," *Nature*, vol. 529, no. 7586, pp. 307–315, 2016.

[38] P. Broz, T. Ruby, K. Belhocine et al., "Caspase-11 increases susceptibility to Salmonella infection in the absence of caspase-1," *Nature*, vol. 490, no. 7419, pp. 288–291, 2012.

[39] G. Gorfu, K. M. Cirelli, M. B. Melo et al., "Dual role for inflammasome sensors NLRP1 and NLRP3 in murine resistance to Toxoplasma gondii," *MBio*, vol. 5, no. 1, 2014.

[40] O. Gross, H. Poeck, M. Bscheider et al., "Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence," *Nature*, vol. 459, no. 7245, pp. 433–436, 2009.

[41] N. Singh, M. Inoue, R. Osawa, M. M. Wagener, and M. L. Shinohara, "Inflammasome expression and cytomegalovirus viremia in critically ill patients with sepsis," *Journal of Clinical Virology*, vol. 93, pp. 8–14, 2017.

[42] A. Asmussen, K. Fink, H. J. Busch et al., "Inflammasome and toll-like receptor signaling in human monocytes after successful cardiopulmonary resuscitation," *Critical Care*, vol. 20, no. 1, p. 170, 2016.

[43] J. N. Ibrahim, I. Jeru, J. C. Lecron, and M. Medlej-Hashim, "Cytokine signatures in hereditary fever syndromes (HFS)," *Cytokine & Growth Factor Reviews*, vol. 33, pp. 19–34, 2017.

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Research Article

A Sustained Reduction in Serum Cholinesterase Enzyme Activity Predicts Patient Outcome following Sepsis

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Early sepsis identification is of paramount importance for an effective therapy and the patient outcome; however, a suitable prognostic biomarker is lacking. Anti-inflammatory nonneuronal cholinergic signaling modulates the magnitude of an immune response. Serum cholinesterase (BChE), an enzyme that hydrolyzes acetylcholine, plays an important role during inflammatory response and serves as an accurate index of cholinergic activity. BChE activity was measured in septic patients using a point-of-care system, and levels of conventional inflammatory markers and the disease severity scores were obtained. We observed a strong, sustained reduction in BChE activity in patients who died within a 90-day observation period, as compared to survivors. Reduced BChE activity when measured at the ICU admission effectively differentiated between the 90-day survivor and the nonsurvivor patient groups. We estimated a critical BChE level of 1.661 kU/L (CI 0.5–0.8, 94% sensitivity, 48% specificity, AUC 0.7) to best predict patient outcome providing a benchmark criterion for early detection of potentially fatal sepsis measured at the admission. This finding suggests that the BChE activity, used in combination with the laboratory tests, clinical examination, and the disease severity scoring, could serve to identify high-risk patients at the ICU admission, the most critical time point in the sepsis treatment.

1. Introduction

Sepsis, an infection complicated by acute organ dysfunction, is the major cause of death in the intensive care units, estimated to be far over 19 million cases per year worldwide [1]. Various factors, including the primary site of infection, the type of pathogen involved, the severity of the acute organ dysfunction, the primary health status, or the delay until the septic patient received the initial therapy, are decisive factors in the clinical appearance, severity, and prognosis of sepsis.

Despite improved modern critical care therapy including an early treatment initiation, support of failing organs, and treatment of the underlying infection, mortality lies between 20 and 30% in the last decade [2]. Early detection of the disease onset is therefore critical for the recognition of complex cases and high-risk patients who require immediate intensive medical care and appropriate therapy.

Various prognostic and diagnostic biomarkers for sepsis have been proposed in the literature [3]. Nevertheless, none of them show sufficient predictive power to be routinely employed in the clinical practice. Sepsis is an inflammatory response to multiple and extremely diverse factors. Therefore, identifying a single sepsis biomarker remains a challenge for critical care medicine.

Disease severity scoring systems (e.g., APACHE II, SOFA, and SAPS II) represent a quick and powerful complement to the laboratory biomarkers routinely used in the clinical setting [4, 5]. Clinical scores are a quick, noninvasive, and reliable assay used for patient stratification and outcome assessment in the ICU [6]. However, most of the scoring

systems require a great amount of diverse patient data including laboratory and organ function tests, as well as a detailed patient history. In some cases, not all data is readily available at the most critical time point: the admission of a patient to the ICU with suspected septic onset.

The cholinergic system plays an important role in maintaining and modulating an adequate immune response upon an inflammatory episode. Cholinergic activity, acting extrasynaptically (nonneuronal acetylcholine), has been proposed to be a major mediator of the neuroimmune response to inflammation in a classical feedback reflex manner [7, 8]. Cholinergic activity is able to modulate the appropriate magnitude of an immune response upon an emerging inflammatory challenge [9–12]. The immune response might therefore be affected by the cholinergic system, acting anti-inflammatory.

Serum cholinesterase (butyrylcholinesterase (BChE)) is an enzyme that hydrolyzes acetylcholine [13]. BChE is synthetized in the liver and has conventionally been used as a liver function test. Our previous work, and that of others, has proposed that the enzymatic activity of the BChE might accurately reflect the nonneuronal cholinergic activity during systemic inflammation [14–20]. By using a point-of-care-testing (POCT) system, we have previously shown a rapid and strong reduction in the BChE activity following an inflammatory challenge [21]. Here, we tested whether the measured BChE activity, obtained from septic patients at the admission to the ICU, might predict the 90-day survival and therefore be used as an early assay for the septic patient outcome.

2. Materials and Methods

2.1. Study Design. This work represents a secondary analysis of the prospective observational study, powered for the detection of mycoses in patients with septic shock [22]. The study was performed at the intensive care unit of the Heidelberg University Hospital. The Ethics Committee of the Medical Faculty at the Heidelberg University approved this study (file number: S-097/2013). All patients or their legal designees gave informed consent. Patients included in this study were diagnosed septic according to the criteria described in the Surviving Sepsis Campaign: International Guidelines for Managements of Severe Sepsis and Septic Shock 2012 [23]. Exclusion criterion for this study was an extensively disrupted liver function with positive results in three or more of the following tests: aspartate aminotransferase (ASAT) > 100 U/L, alanine aminotransferase (ALAT) > 100 U/L, gamma glutamyl transferase (GGT) > 100 U/L, alkaline phosphatase (AP) > 200 U/L, total bilirubin > 2 mg/dL, and international normalized ratio (INR) > 1.3. Out of 50 recruited patients, 9 patients had documented extensive liver function disruption and were therefore excluded from the study. This resulted in a study population of 41 patients. Basic demographic data, site of infection, and the patient outcome are listed in Table 1.

Table 1: Study population demographics and clinical data.

Patient data	
Number of patients	41
Age (years)*	66 (62–77)
Gender (male/female)	29/12
Septic focus	
Gastrointestinal tract	37 (90%)
Lung	8 (20%)
Genitourinary tract	1 (2%)
Other	2 (5%)
Surgery	
Patients subjected to the surgical revision	24 (59%)
Length of stay	
Days in the ICU*	9 (4–18)
Days in the IMC*	6 (0–14)
Days in the high dependence units (IMC + ICU)*	18 (9–37)
Days in the hospital*	40 (19–62)

ICU: intensive care unit; IMC: intermediate care unit. *Median with interquartile range.

2.2. Measurements. Blood samples used in this study were gathered at the time point of the sepsis detection, followed by blood samples collected 1, 2, 7, 14, 21, and 28 days later. Serum cholinesterase enzyme activity was measured by using the ChE check (Securetec Detektions-Systeme AG, Neubiberg, Germany; In-Vitro-Diagnostics Guideline 98/79/EG; DIN EN ISO 18113-2 and DIN EN ISO 18113-3) point-of-care-testing (POCT) device by following the manufacturer's instructions, as previously described in Zivkovic et al. [14]. Enzyme activity is measured in kU/L. The analysis of conventional inflammation biomarkers white blood cell count (WBCC), C-reactive protein (CRP), and procalcitonin (PCT) was conducted according to the standardized protocols of the central laboratory of the clinic. Plasma concentrations of interleukins IL-4, IL-6, and IL-10, tumor necrosis factor alpha (TNF-α), and midrange proadrenomedullin (MR-proADM) were measured according to the manufacturer's instructions as previously described [22]. Disease severity of septic patients was assessed at the ICU by obtaining APACHE II (Acute Physiology and Chronic Health Evaluation II), SOFA (Sequential Organ Failure Assessment), and SAPS II (Simplified Acute Physiology Score) scores. Patients were routinely scored at the above described time points for blood samples.

2.3. Statistical Analysis. The data were electronically gathered and stored by using Excel (Microsoft Corp., Redmond, WA). Data analysis was performed by using GraphPad Prism 6 for Mac (GraphPad Software, La Jolla California, USA, http://www.graphpad.com) and SPSS (version 21.0; SPSS Inc., Chicago, IL, USA). The optimal cutoff value estimation was performed by using Youden index. Data are presented as median with interquartile range (IQR). D'Agostino and Pearson omnibus normality test was used to verify the Gaussian distribution of the study groups. Statistical significance between the patient groups was tested using Mann–Whitney U test. A p value < 0.05 indicated statistical significance.

3. Results

We set the 90-day survival as a measure of the patient outcome following sepsis detection. Septic patients were divided in two groups: those who survived 90 days (survivors, 25 patients) and patients who did not survive 90 days following sepsis (nonsurvivors, 16 patients). Using the POCT, BChE enzyme activity was measured at the starting time point and on days 1, 2, 7, 14, 21, and 28 following the clinical onset of sepsis. As compared to the previously reported normal range [14], the BChE enzyme activity was markedly reduced in all septic patients during the observation period ($p = 0.0001$, Mann–Whitney U test), and the measured BChE activity in nonsurvivors was notably lower than that of the surviving patients for the duration of 28 days ($p < 0.01$, Mann–Whitney U test, Figures 1(a) and 1(b), Supplementary Figure 1, Supplementary Table 1). To be able to compare the BChE activity changes between patients, the enzyme activity was normalized to the initial value, obtained at the time point of the clinical onset of the sepsis. The analysis of the normalized BChE activity enabled the assessment of the amount and the rate of the enzyme activity changes during the observation period. The normalized BChE activity decreased within the first two days in both patient groups. During the following observation period, the normalized BChE activity of the survivors recovered, whereas the normalized BChE activity of the nonsurvivors remained strongly reduced (Figures 1(c) and 1(d), Supplementary Table 1). However, no statistical significance could be observed between the two patient groups. Concurrently, measured activity of the conventional inflammation biomarkers, CRP (Figures 1(e) and 1(f), Supplementary Table 1) and WBCC (Figures 1(g) and 1(h), Supplementary Table 1), showed an initial increase in activity, peaking two days after the clinical onset of the sepsis. No statistical difference between the surviving and the nonsurviving patients was observed.

Furthermore, we tested the activity pattern of PCT, a sepsis biomarker showing elevated activity during bacterial and fungal but not viral infections. Although increased PCT activity was initially observed in nonsurvivors, no differences were seen at the later time points (Figures 2(a) and 2(b), Supplementary Table 1). Significantly higher activity levels of MR-proADM, a novel sepsis biomarker, were observed in nonsurvivors in later time points starting 21 days following clinical onset of sepsis ($p < 0.05$, Mann–Whitney U test). However, MR-proADM activity levels could not discriminate between the surviving and the nonsurviving patients, when measured during the initial observation period (Figures 2(c) and 2(d), Supplementary Table 1). The activity pattern of the proinflammatory cytokines IL-6 and TNF-α as well as the anti-inflammatory cytokines IL-4 and IL-10 showed comparable activity patterns without a significant difference between the two patient groups in the initial observation period. The difference in the cytokine activities could be observed in the later time points after the clinical onset of the disease (after 28 days for IL-6, $p < 0.05$, Figures 2(e) and 2(f), Supplementary Table 1; after 14 days for TNF-α, $p < 0.05$, Figures 2(g) and 2(h), Supplementary Table 1; after 21 days for IL-4, $p < 0.01$, Supplementary Figure 2a, and 2b, Supplementary Table 2; and after 7 days for IL-10, $p < 0.05$, Supplementary Figure 2c, and 2d, Supplementary Table 2; Mann–Whitney U test). Thus, none of these conventional biomarkers could early predict patient survival at the 90-day period.

Next, we analyzed how well the measured BChE activity predicts the outcome of the septic patients. The 90-day survival of 61% following the clinical onset of the sepsis served as an independent long-term outcome parameter (Figure 3(a)). We observed that measured BChE enzyme activity could predict the survival of the septic patients. The receiver operator characteristic (ROC) curve analysis revealed an area under the curve (AUC) of 0.7. Further analysis rendered an optimal cutoff value of 1.661 kU/L for the measured BChE enzyme activity with 94% sensitivity and 48% specificity (Figure 3(b)). This finding was verified by plotting the Kaplan–Meier survival curves from septic patients with the initial BChE activity greater than the cutoff value and those with the initial BChE activity below the optimal cutoff value (Figure 3(c)). In addition, the ROC curve analysis of the conventional and novel sepsis biomarkers could poorly predict the outcome of the septic patients when measured at the initial time point (Supplementary Figure 3). We further examined the relationship between the patient outcome and the initially measured inflammation parameters (BChE, CRP, WBCC, PCT, MR-proADM, TNF-alpha, IL-4, IL-6, and IL-10) by conducting the U test and the full stepwise logistic regression analysis. Results of the U test ($p = 0.04$) and the stepwise logistic regression analysis ($p = 0.03$, likelihood ratio test) identified BChE as a sole independent prognostic factor for the patient outcome. Furthermore, we performed separate stepwise logistic regression analyses for three parameter subgroups. Separate analysis of the first subgroup (BChE, PCT, and MR-proADM) identified BChE enzyme activity as an independent prognostic factor for patient outcome ($p = 0.02$; likelihood ratio test). IL-10 was identified in the second subgroup (TNF-alpha, IL-10, and CRP) as an independent prognostic factor for patient outcome ($p = 0.04$; likelihood ratio test). In the third subgroup of factors (IL-4, IL-6, and WBCC), both IL-4 ($p = 0.02$, likelihood ratio test) and IL-6 ($p = 0.045$, likelihood ratio test) could be identified as independent prognostic factors for patient outcome.

BChE is synthetized in the liver, so impaired liver function would result in reduced BChE activity. To verify that reduced BChE activity is not caused due to liver dysfunction, we analyzed the liver function and found no impairment in septic patients included in the study (Supplementary Figure 4, Supplementary Table 3).

To assess the patient outcome following sepsis, we concurrently calculated the APACHE II and SOFA as well as SAPS II scores (Figure 4, Supplementary Table 1) for the survivor and the nonsurvivor patient groups. A difference between the two patient groups was observed 21 days following the clinical sepsis detection in all disease severity scores (APACHE II, $p < 0.05$, Figures 4(a) and 4(d), Supplementary Table 1; SOFA, $p < 0.01$, Figures 4(b) and 4(e), Supplementary Table 1; and SAPS II, $p < 0.001$; Mann–Whitney U test). However, the disease severity scores could not discriminate

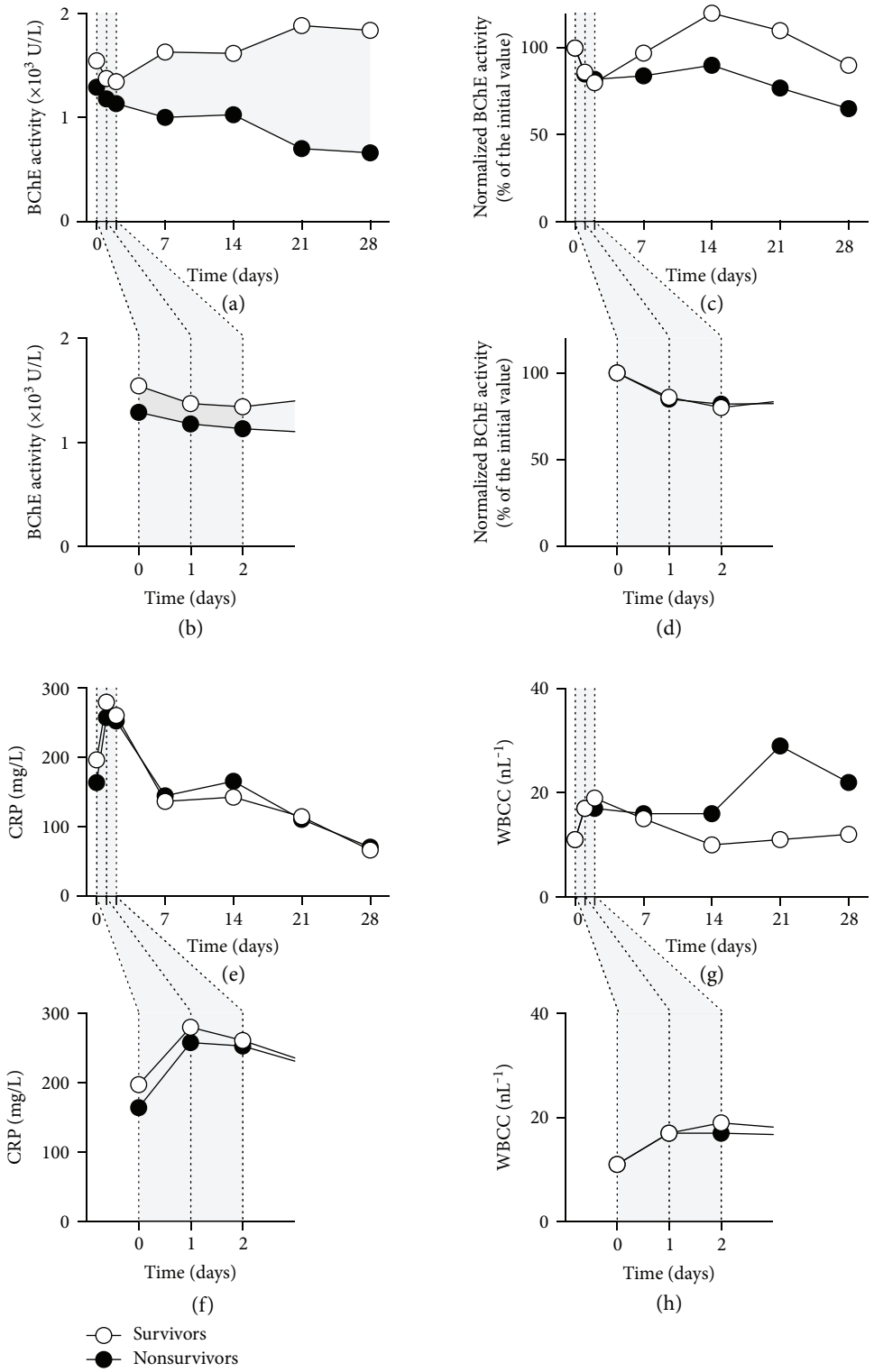

Figure 1: A sustained reduction in BChE enzyme activity identifies 90-day nonsurvivors following sepsis. BChE (a–d), CRP (e, f), and WBCC (g, h) measurements obtained from patient blood samples taken at the time point of the sepsis detection (0) and 1, 2, 7, 14, and 28 days later in patients surviving 90 days (open circles) and in nonsurvivors (closed circles). BChE enzyme activity is plotted as a raw value (a) and normalized to day 0 (initial value, c). Magnified time lines (b, d, f, h) show the measurements obtained during the first two days following sepsis detection. Dark gray-shaded areas indicate time periods where the statistical significance between the two patient groups occurs ($p < 0.01$, Mann–Whitney U test). Data are medians. BChE: butyrylcholinesterase; CRP: C-reactive protein; WBCC: white blood cell count.

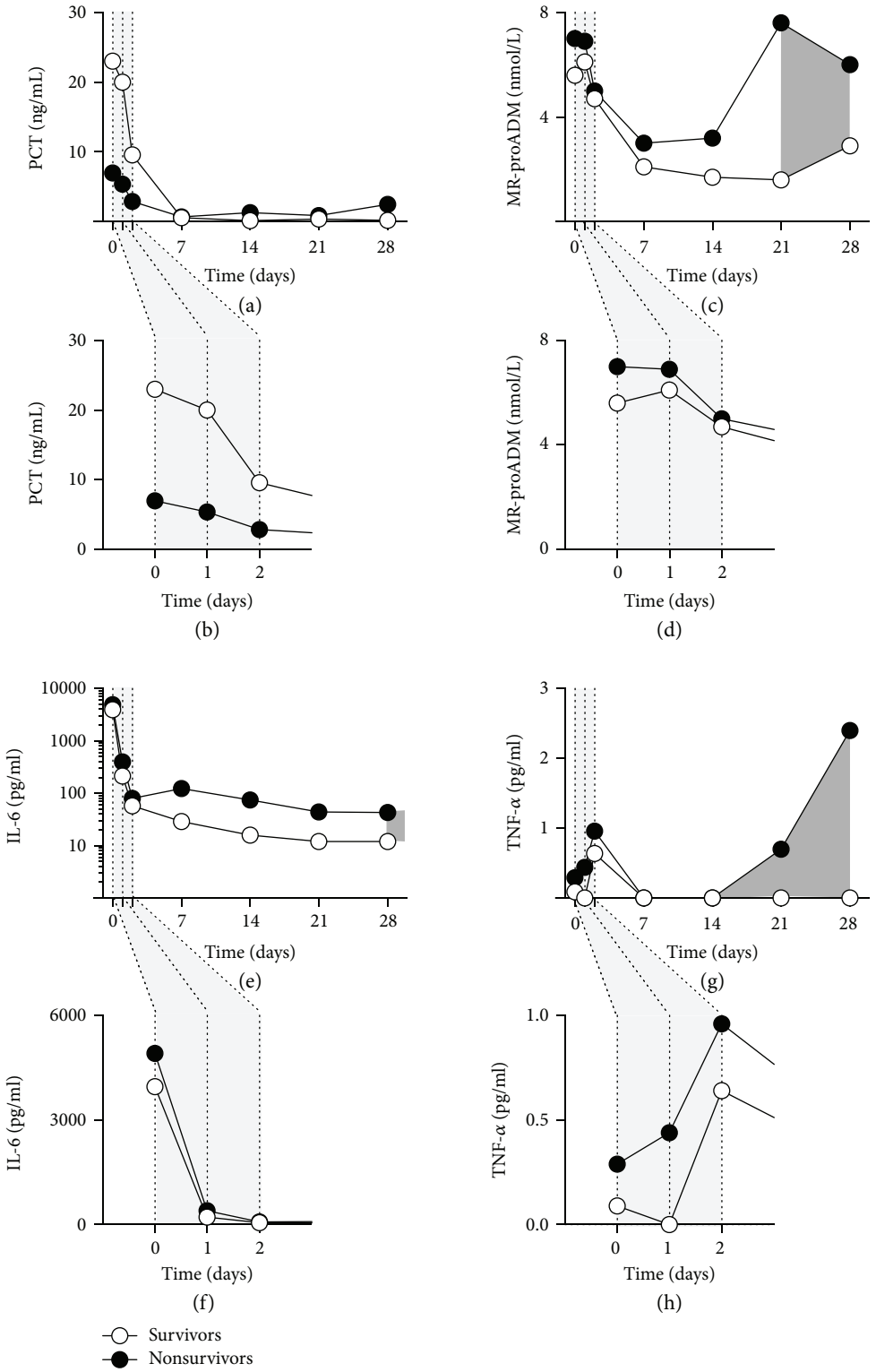

Figure 2: Activity pattern of the inflammatory biomarkers in patients with sepsis. Activity of PCT (a, b), MR-proADM (c, d), IL-6 (e, f), and TNF-α (g, h) was measured at the time point of sepsis detection (0) as well as 1, 2, 7, 14, 21, and 28 days later in patients who survived (open circles) and in those who died within 90 days (closed circles). Magnified time lines (b, d, f, h) represent measurements obtained within the first two days after the sepsis detection. Dark gray-shaded areas indicate time periods with the statistical difference between the patient groups ($p < 0.05$, Mann–Whitney U test). Data points are medians. PCT: procalcitonin; MR-proADM: midrange proadrenomedullin; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha.

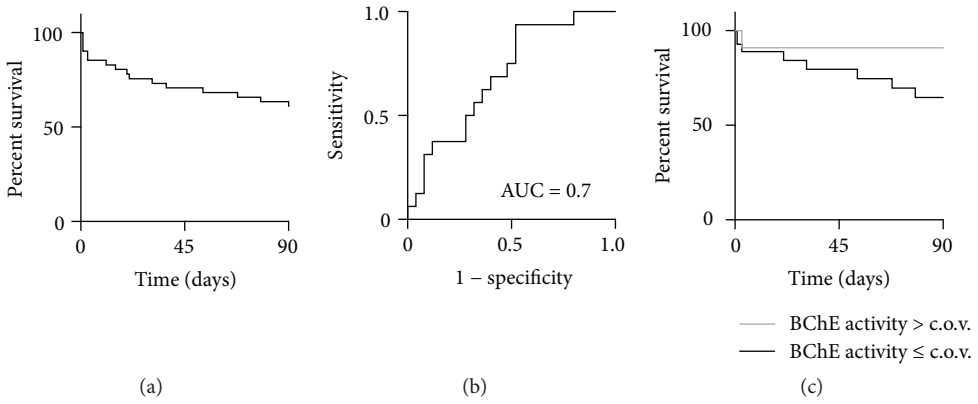

Figure 3: Measured BChE enzyme activity predicts the patient outcome following sepsis. (a) Kaplan–Meier curve shows the survival analysis of septic patients included in this study. (b) ROC curve analysis for the BChE activity measured at the time point of the clinical detection of sepsis revealed an AUC of 0.7 with the optimal cutoff value of 1.661×10^3 U/L (CI 0.5–0.8, 94% sensitivity, 48% specificity). (c) A comparison of the Kaplan–Meier curves stratified according to the cutoff value for the measured BChE enzyme activity, as shown in (b). BChE: butyrylcholinesterase; ROC: receiver operating characteristic; AUC: area under the curve; CI: confidence interval; c.o.v.: cutoff value.

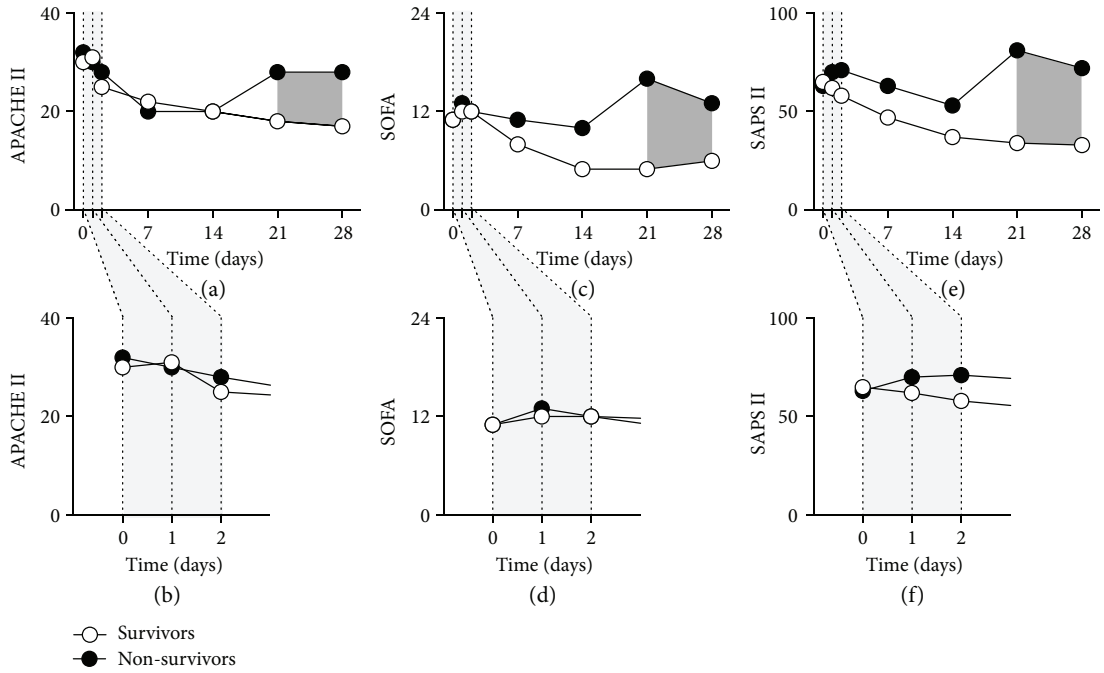

Figure 4: Disease severity scores predict outcome in patients with sepsis. APACHE II (a, b), SOFA (c, d), and SAPS II (e, f) scores were calculated from patients surviving sepsis (open circles) and from those who did not survive 90 days following sepsis detection (closed circles). Disease severity scores were obtained at the time point of the sepsis diagnosis and repeated 1, 2, 7, 14, 21, and 28 days later. Magnified time lines (b, d, f) show the disease severity scores obtained within the first two days following sepsis detection. Dark gray-shaded areas indicate the time points where the statistical significance could be observed ($p < 0.05$ for APACHE II, $p < 0.01$ for SOFA, and $p < 0.001$ for SAPS II; Mann–Whitney U test). Data points are medians. APACHE II: Acute Physiology and Chronic Health; SOFA: Sequential Organ Failure Assessment; SAPS II: Simplified Acute Physiology Score.

the nonsurvivors from the survivors when obtained at the initial time point.

4. Discussion

The present study highlights the validity and prognostic power of BChE measurements for early detection of high-risk, life-threatening sepsis. Reduced activity of the BChE, when measured at the clinical onset of sepsis, mirrors the activity changes of both conventional (CRP, WBCC, PCT) and novel (IL-4, IL-6, IL-10, TNF-α, MR-proADM) inflammation biomarkers and was validated as a biomarker in the early detection of sepsis by its correlation with several disease severity scores used for patient outcome analysis (APACHE

II, SOFA, and SAPS II). Surprisingly, the BChE assessment at the onset of sepsis proved more effective than these disease severity scores in discriminating between the surviving and the nonsurviving patient groups making it a valuable biomarker for the early detection of high-risk sepsis patients.

In accordance with our previous studies, the reduction in BChE activity was observed as early as 1-2 hours following the inflammatory onset [21]. Moreover, a sustained BChE activity reduction, observed in nonsurviving patients, represents a novel red flag warning indicator in the critical care setting, allowing simple and rapid detection of the high-risk septic patients.

Septic patients included in the study showed elevated CRP and WBCC levels, which corresponded to the well-described pathophysiology following an immune response [24–30]. However, the initial measurements of these inflammatory biomarkers failed to distinguish between the survivor and the nonsurvivor patient groups in this study.

The levels of proinflammatory cytokines IL-6 and TNF-α, suggested to be effective in sepsis detection and mortality prediction in severe sepsis [31–36], showed altered, abnormal levels as expected throughout the observation period; however, these measurements during the initial 48 hours of observations could not discriminate the survivor and the nonsurvivor patient groups. We interpret this finding to be either due to the delayed abnormalities in cytokine peak activity (72 hours) [37], as compared to the earlier change in BChE activity, or due to the rather high variability of the obtained cytokine measurements. Moreover, separate subgroup stepwise logistic regression analysis identified IL-4, IL-6, and IL-10 as independent predictors for patient outcome, when measured at the initial time point. However, this finding could not be observed in other concurrently performed tests (U test, full stepwise regression analysis, Mann–Whitney U test). We interpret this observation to be presumably due to a low number of study patients and a high variability of the obtained interleukin measurements. Conducting a study with a larger population would presumably help overcome the observed discrepancy; however, such high variability suggests that cytokine tests will not provide a reliable biomarker assay for individual patient prognosis.

Our ROC analysis, identifying the optimal BChE cutoff value of the measured BChE enzyme activity at the time point corresponding to the clinical onset of sepsis, suggests that a single bedside BChE measurement could early identify a high-risk patient. Bedside measurement of BChE activity might help reduce the time delay between the initial clinical presentation of the patient with the suspected sepsis and the first therapeutic decisions, when conventional laboratory and diagnostic tests (e.g., chest X-ray and microbiology results) and clinical scores are not readily available. This is highly relevant for ICU clinicians who are often confronted with a fulminant course of the disease, requiring immediate action.

The AUC testing showed a reasonable accuracy for the BChE activity (0.7), which could be explained by the small group size in this study. The optimal cutoff value for the 90-day survival compares to the previously described BChE levels, observed upon the onset of the severe systemic inflammation [14]. Our previous study showed even stronger reduction of the normalized BChE activity upon systemic inflammation than nonnormalised data [21]; however, we did not observe this effect in the present study. Probable reason could be that the exact time point of the clinical diagnosis of the sepsis (and the time point of the initial BChE measurement) differed from the actual time point of the primary inflammation. The missed time interval between the genuine inflammation and the clinical onset of sepsis presumably led to a failed initial BChE measurement and the resulting initial activity reduction. This hypothesis could be further supported by the finding that the initially measured levels of the conventional inflammatory biomarkers (CRP, WBCC, PCT, IL-6, and TNF-α) showed markedly elevated activity, which are regularly described to peak at the earliest 24 hours following the inflammatory onset. This observation might therefore suggest that the proposed optimal cutoff value might be somewhat higher. Further study would be needed to answer this question.

MR-proADM, a peptide with immune modulating, metabolic, and vasoactive properties [38], has been shown to be an effective prognostic marker in sepsis [39] and septic shock [40] with a comparable and even superior prognostic value for sepsis relative to the conventional inflammatory biomarkers (CRP, PCT, and IL-6), as well as to the disease severity scores (APACHE II and SAPS) [39]. We observed a comparable pattern in BChE and MR-proADM activity when compared between the survivor and the nonsurvivor patient groups, further validating the efficacy of the BChE in predicting survival of patients with sepsis.

Disease severity scores are well-established clinical assays for the patient outcome analysis [6, 41–43]. In the present study, the difference between the two patient groups was revealed by the score calculations; however, these scores failed to discriminate the survivors from the nonsurvivors at the starting time point. These clinical scores are efficient tools in predicting outcome; however, they require documenting multiple and diverse datasets. The datasets are in most cases readily available; however, in some cases, a particular set of data might not be accessible, delaying or making the scoring impossible. By using a POCT system for a single BChE measurement, the results of an equally efficient outcome assessment tool are readily available at the bedside.

Why do nonsurviving septic patients show continuous and markedly lower levels of the serum cholinesterase enzymatic activity as compared to their surviving counterparts? Nonneuronal cholinergic activity is known to participate in the immune response [44]. The central nervous system (CNS) receives afferents from the immune system via both the humoral and the neural pathways [45, 46]. The humoral route uses the active transport of the proinflammatory cytokines through the blood-brain barrier, carrying the information about the commencing inflammatory process in the periphery. The neural immune pathway to the CNS from affected organs prevails when cytokine levels are low, providing early alert information of mild to moderate inflammation. This neural inflammatory signaling, together with pain and injury signals from the periphery, operates at a

much lower detection threshold and reaches the CNS much quicker than the humoral pathway [12, 47]. The vagus nerve, a cholinergic neuron innervating most of the organs, mediates the neuroimmune response. The extrasynaptic cholinergic activity might therefore play a crucial role in both phases of the neuroimmune response: a quick and effective detection of an inflammatory challenge in the early phase but also in the process of maintaining the equilibrium of the neuroimmune response in the later phase of the severe inflammation. Therefore, the extrasynaptic activity of the cholinergic system might be of great importance during both early and late phases of the inflammatory response.

How does the enzyme activity of the serum cholinesterase relate to the cholinergic activity during an inflammatory episode? Several hypotheses might explain the observed phenomenon. Firstly, cholinergic neurotransmission is continuously active in a feedback response manner, maintaining physiological homeostasis. An inflammatory challenge would immediately cause cholinergic activation, causing a counteracting immune reaction [8]. The neuroimmune loop might act much quicker than the conventional immune reaction [48, 49]. The role of the reduced serum cholinesterase might mirror a neuroimmune setting where increased cholinergic activity is required. In the later time point, when the inflammation recedes and the organism recovers, cholinergic activity and the resulting levels of the serum cholinesterase will slowly increase, returning to the baseline. Such homeostatic regulation of BChE would presumably not take place in the continuously challenged and decompensated system of the nonsurvivor, where constantly low levels of the BChE activity mirror a decompensated organism which eventually leads to severe organ failure and death. The question whether low levels of BChE are caused by low levels of the extrasynaptically available enzyme substrate, acetylcholine, or a separate yet unknown mechanism reduces the BChE levels remains an open question. In the presence of low BChE activity, elevated levels of the extrasynaptic cholinergic activity would ensue, producing an anti-inflammatory response needed during the severe systemic inflammation. At this moment, it is not possible to provide a plausible answer whether reduced BChE activity results from the presumably reduced level of the nonneuronal acetylcholine or is actually an underlying cause. Further studies would be needed to address these questions.

Since serum cholinesterase is an enzyme synthetized in the liver [50, 51], the activity of the BChE is affected by disrupted liver function [52]. A clear limitation for using BChE enzyme activity as a biomarker for potentially fatal sepsis is patients with dysfunctional livers. The results obtained from these patients could not reliably be interpreted. Thus, the authors recommend against using this assay in patients with severe dysfunction of the liver.

A further limitation of this study is the low number of included patients. However, even with the low study sample, the described test demonstrated high sensitivity, particularly in the initial time period, as compared to the benchmark methods, suggesting a rapid, effective, and simple patient outcome assay. A larger, possibly multicenter, study would certainly be needed to validate our findings.

In summary, a single measurement of the BChE enzyme activity may serve as the simplest way to best predict patient outcome when performed at the initial time point. Moreover, the BChE activity correlates with the activity pattern of the conventional inflammatory biomarkers. The observed difference between the 90-day survivors and the nonsurvivors obtained by calculating the disease severity scores, a benchmark tool for the outcome analysis in the clinical setting, further verified the advantage of using the POCT method for a rapid patient outcome screening.

5. Conclusions

A single bedside measurement of the serum cholinesterase activity might be used to assess the 90-day patient outcome following sepsis. A suggested optimal cutoff value 1.661 kU/L, when obtained at the time point of the clinical onset of sepsis, might predict patient survival with 94% sensitivity and 48% specificity. This assay provides a rapid and simple bedside test which might be used for an early, simple, and effective identification of high-risk septic patients.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Aleksandar R. Zivkovic, Sebastian O. Decker, Karsten Schmidt, Markus A. Weigand, Stefan Hofer, and Thorsten Brenner participated in the design of the study. Aleksandar R. Zivkovic wrote the paper. Annette Sigl carried out the measurements. Aleksandar R. Zivkovic and Anne C. Zirnstein performed the data analysis. Aleksandar R. Zivkovic, Sebastian O. Decker, and Thorsten Brenner participated in the coordination of the study. All authors read and approved the final paper. Aleksandar R. Zivkovic and Sebastian O. Decker contributed equally to this work.

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Supplementary Materials

Supplementary 1. Table 1: result summary.

Supplementary 2. Table 2: result summary for Supplementary Figure 2.

Supplementary 3. Table 3: result summary for Supplementary Figure 4.

Supplementary 4. Figure 1: the enzyme activity of the BChE in septic patients. Individual measurements (a, b) and median values (c, d) of the BChE enzyme activity obtained from 90-day survivors (open circles; a, c) and from patients who did not survive 90 days (closed circles, b, d) are obtained at the time point of the sepsis detection, 1, 2, 7, 14, 21, and 28 days later. Lines in (a) and (b) represent medians. Error bars in (c) and (d) are interquartile range.

Supplementary 5. Figure 2: activity pattern of the anti-inflammatory cytokines in septic patients. Activity of IL-4 (a, b) and IL-10 (c, d) was measured at the time point of sepsis detection (0) as well as 1, 2, 7, 14, 21, and 28 days later in patients who survived (open circles) and in those who died within 90 days (closed circles). Graphs (b) and (d) represent magnified time lines of the corresponding measurements obtained within the first two days after the sepsis detection. Data points are medians. IL-4: interleukin 4; IL-10: interleukin 10.

Supplementary 6. Figure 3: ROC curve analysis for the inflammatory biomarkers and disease severity scores. The ROC curves for the conventional inflammation biomarkers CRP, WBCC, and PCT are shown in panel (a). The ROC curves for MR-proADM, TNF-α, IL-4, IL-6, and IL-10 are shown in panel (b). The ROC analysis curves of the disease severity scores APACHE II, SOFA, and SAPS II are shown in panel (c). ROC: receiver operating characteristic; AUC: area under the curve; CRP: C-reactive protein; WBCC: white blood cell count; PCT: procalcitonin; MR-proADM: midrange proadrenomedullin; IL-4: interleukin 4; IL-6: interleukin 6; IL-10: interleukin 10; TNF-α: tumor necrosis factor alpha; APACHE II: Acute Physiology and Chronic Health; SOFA: Sequential Organ Failure Assessment; SAPS II: Simplified Acute Physiology Score.

Supplementary 7. Figure 4: liver function tests in septic patients. Median values of the ASAT, ALAT, GGT, and AP activities obtained from 90-day survivors (white) and from 90-day nonsurvivors (gray) are shown in the bar histogram (a). Histogram (b) shows median bilirubin and INR values obtained from the two patient groups. Shown data represent medians of the pooled values, collected at the previously described time points. Error bars are interquartile range. ASAT: aspartate aminotransferase; ALAT: alanine aminotransferase; AP: alkaline phosphatase; GGT: gamma glutamyl transferase; INR: international normalized ratio.

References

[1] N. K. J. Adhikari, R. A. Fowler, S. Bhagwanjee, and G. D. Rubenfeld, "Critical care and the global burden of critical illness in adults," *The Lancet*, vol. 376, no. 9749, pp. 1339–1346, 2010.

[2] D. C. Angus, W. T. Linde-Zwirble, J. Lidicker, G. Clermont, J. Carcillo, and M. R. Pinsky, "Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care," *Critical Care Medicine*, vol. 29, no. 7, pp. 1303–1310, 2001.

[3] C. Pierrakos and J. L. Vincent, "Sepsis biomarkers: a review," *Critical Care*, vol. 14, no. 1, article R15, 2010.

[4] N. O. Ghanem-Zoubi, M. Vardi, A. Laor, G. Weber, and H. Bitterman, "Assessment of disease-severity scoring systems for patients with sepsis in general internal medicine departments," *Critical Care*, vol. 15, no. 2, article R95, 2011.

[5] A. D. Sekulic, S. V. Trpkovic, A. P. Pavlovic, O. M. Marinkovic, and A. N. Ilic, "Scoring systems in assessing survival of critically ill ICU patients," *Medical Science Monitor*, vol. 21, pp. 2621–2629, 2015.

[6] J. L. Vincent and R. Moreno, "Clinical review: scoring systems in the critically ill," *Critical Care*, vol. 14, no. 2, p. 207, 2010.

[7] U. Andersson and K. J. Tracey, "Neural reflexes in inflammation and immunity," *Journal of Experimental Medicine*, vol. 209, no. 6, pp. 1057–1068, 2012.

[8] C. J. Czura and K. J. Tracey, "Autonomic neural regulation of immunity," *Journal of Internal Medicine*, vol. 257, no. 2, pp. 156–166, 2005.

[9] U. Andersson and K. J. Tracey, "Reflex principles of immunological homeostasis," *Annual Review of Immunology*, vol. 30, no. 1, pp. 313–335, 2012.

[10] K. J. Tracey, "Reflex control of immunity," *Nature reviews Immunology*, vol. 9, no. 6, pp. 418–428, 2009.

[11] K. J. Tracey, "Physiology and immunology of the cholinergic antiinflammatory pathway," *The Journal of Clinical Investigation*, vol. 117, no. 2, pp. 289–296, 2007.

[12] K. J. Tracey, "The inflammatory reflex," *Nature*, vol. 420, no. 6917, pp. 853–859, 2002.

[13] S. Darvesh, D. A. Hopkins, and C. Geula, "Neurobiology of butyrylcholinesterase," *Nature Reviews Neuroscience*, vol. 4, no. 2, pp. 131–138, 2003.

[14] A. R. Zivkovic, K. Schmidt, A. Sigl, S. O. Decker, T. Brenner, and S. Hofer, "Reduced serum butyrylcholinesterase activity indicates severe systemic inflammation in critically ill patients," *Mediators of Inflammation*, vol. 2015, Article ID 274607, 11 pages, 2015.

[15] G. M. do Carmo, L. Z. Crivellenti, N. B. Bottari et al., "Butyrylcholinesterase as a marker of inflammation and liver injury in the acute and subclinical phases of canine ehrlichiosis," *Comparative Immunology, Microbiology and Infectious Diseases*, vol. 43, pp. 16–21, 2015.

[16] Q. H. Zhang, A. M. Li, S. L. He et al., "Serum total cholinesterase activity on admission is associated with disease severity and outcome in patients with traumatic brain injury," *PLoS One*, vol. 10, no. 6, article e0129082, 2015.

[17] K. Distelmaier, M. P. Winter, K. Rützler et al., "Serum butyrylcholinesterase predicts survival after extracorporeal membrane oxygenation after cardiovascular surgery," *Critical Care*, vol. 18, no. 1, article R24, 2014.

[18] G. A. Reid, N. Chilukuri, and S. Darvesh, "Butyrylcholinesterase and the cholinergic system," *Neuroscience*, vol. 234, pp. 53–68, 2013.

[19] N. Lampón, E. F. Hermida-Cadahia, A. Riveiro, and J. C. Tutor, "Association between butyrylcholinesterase activity and low-grade systemic inflammation," *Annals of Hepatology*, vol. 11, no. 3, pp. 356–363, 2012.

[20] K. Ofek, K. S. Krabbe, T. Evron et al., "Cholinergic status modulations in human volunteers under acute inflammation," *Journal of Molecular Medicine*, vol. 85, no. 11, pp. 1239–1251, 2007.

[21] A. R. Zivkovic, J. Bender, T. Brenner, S. Hofer, and K. Schmidt, "Reduced butyrylcholinesterase activity is an early indicator of

trauma-induced acute systemic inflammatory response," *Journal of Inflammation Research*, vol. 9, pp. 221–230, 2016.

[22] S. O. Decker, A. Sigl, C. Grumaz et al., "Immune-response patterns and next generation sequencing diagnostics for the detection of mycoses in patients with septic shock—results of a combined clinical and experimental investigation," *International Journal of Molecular Sciences*, vol. 18, no. 8, article 1796, 2017.

[23] R. P. Dellinger, M. M. Levy, A. Rhodes et al., "Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock," *Critical Care Medicine*, vol. 41, no. 2, pp. 580–637, 2013.

[24] M. B. Pepys and G. M. Hirschfield, "C-reactive protein: a critical update," *Journal of Clinical Investigation*, vol. 111, no. 12, pp. 1805–1812, 2003.

[25] T. Welsch, K. Frommhold, U. Hinz et al., "Persisting elevation of C-reactive protein after pancreatic resections can indicate developing inflammatory complications," *Surgery*, vol. 143, no. 1, pp. 20–28, 2008.

[26] G. P. Castelli, C. Pognani, M. Cita, A. Stuani, L. Sgarbi, and R. Paladini, "Procalcitonin, C-reactive protein, white blood cells and SOFA score in ICU: diagnosis and monitoring of sepsis," *Minerva anestesiologica*, vol. 72, no. 1-2, pp. 69–80, 2006.

[27] A. C. M. Kobold, J. E. Tulleken, J. G. Zijlstra et al., "Leukocyte activation in sepsis; correlations with disease state and mortality," *Intensive Care Medicine*, vol. 26, no. 7, pp. 883–892, 2000.

[28] K. Tschaikowsky, M. Hedwig-Geissing, G. G. Braun, and M. Radespiel-Troeger, "Predictive value of procalcitonin, interleukin-6, and C-reactive protein for survival in postoperative patients with severe sepsis," *Journal of Critical Care*, vol. 26, no. 1, pp. 54–64, 2011.

[29] R. F. Mortensen, "C-Reactive protein, inflammation, and innate immunity," *Immunologic Research*, vol. 24, no. 2, pp. 163–176, 2001.

[30] G. P. Castelli, C. Pognani, M. Meisner, A. Stuani, D. Bellomi, and L. Sgarbi, "Procalcitonin and C-reactive protein during systemic inflammatory response syndrome, sepsis and organ dysfunction," *Critical Care*, vol. 8, no. 4, article R234, 2004.

[31] J. Cohen, "The immunopathogenesis of sepsis," *Nature*, vol. 420, no. 6917, pp. 885–891, 2002.

[32] T. S. Blackwell and J. W. Christman, "Sepsis and cytokines: current status," *British Journal of Anaesthesia*, vol. 77, no. 1, pp. 110–117, 1996.

[33] P. Dama, D. Ledoux, M. Nys et al., "Cytokine serum level during severe sepsis in human IL-6 as a marker of severity," *Annals of Surgery*, vol. 215, no. 4, pp. 356–362, 1992.

[34] C. A. Gogos, E. Drosou, H. P. Bassaris, and A. Skoutelis, "Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options," *The Journal of Infectious Diseases*, vol. 181, no. 1, pp. 176–180, 2000.

[35] A. Oberholzer, S. M. Souza, S. K. Tschoeke et al., "Plasma cytokine measurements augment prognostic scores as indicators of outcome in patients with severe sepsis," *Shock*, vol. 23, no. 6, pp. 488–493, 2005.

[36] F. A. Bozza, J. I. Salluh, A. M. Japiassu et al., "Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis," *Critical Care*, vol. 11, no. 2, pp. R49–R48, 2007.

[37] D. Andaluz-Ojeda, F. Bobillo, V. Iglesias et al., "A combined score of pro- and anti-inflammatory interleukins improves mortality prediction in severe sepsis," *Cytokine*, vol. 57, no. 3, pp. 332–336, 2012.

[38] P. Linscheid, D. Seboek, H. Zulewski, U. Keller, and B. Müller, "Autocrine/paracrine role of inflammation-mediated calcitonin gene-related peptide and adrenomedullin expression in human adipose tissue," *Endocrinology*, vol. 146, no. 6, pp. 2699–2708, 2005.

[39] M. Christ-Crain, N. G. Morgenthaler, J. Struck, S. Harbarth, A. Bergmann, and B. Müller, "Mid-regional pro-adrenomedullin as a prognostic marker in sepsis: an observational study," *Critical Care*, vol. 9, article R816, 2005.

[40] M. V. de la Torre-Prados, A. Garcia-De La Torre, A. Enguix, M. Mayor-Reyes, M. Nieto-González, and A. Garcia-Alcantara, "Mid-regional pro-adrenomedullin as prognostic biomarker in septic shock," *Minerva Anestesiologica*, vol. 82, pp. 760–766, 2016.

[41] W. A. Knaus, E. A. Draper, D. P. Wagner, and J. E. Zimmerman, "APACHE II: a severity of disease classification system," *Critical care medicine*, vol. 13, no. 10, pp. 818–829, 1985.

[42] J. R. Le Gall, S. Lemeshow, and F. Saulnier, "A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study," *JAMA*, vol. 270, no. 24, pp. 2957–2963, 1993.

[43] J. L. Vincent, R. Moreno, J. Takala et al., "The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure," *Intensive Care Medicine*, vol. 22, no. 7, pp. 707–710, 1996.

[44] H. Wang, M. Yu, M. Ochani et al., "Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation," *Nature*, vol. 421, no. 6921, pp. 384–388, 2003.

[45] J. E. Blalock, "The immune system as the sixth sense," *Journal of Internal Medicine*, vol. 257, no. 2, pp. 126–138, 2005.

[46] J. E. Blalock, "The immune system as a sensory organ," *The Journal of immunology*, vol. 132, no. 3, pp. 1067–1070, 1984.

[47] L. V. Borovikova, S. Ivanova, M. Zhang et al., "Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin," *Nature*, vol. 405, no. 6785, pp. 458–462, 2000.

[48] L. E. Goehler, R. P. A. Gaykema, M. K. Hansen, K. Anderson, S. F. Maier, and L. R. Watkins, "Vagal immune-to-brain communication: a visceral chemosensory pathway," *Autonomic Neuroscience: Basic & Clinical*, vol. 85, no. 1-3, pp. 49–59, 2000.

[49] V. A. Pavlov, H. Wang, C. J. Czura, S. G. Friedman, and K. J. Tracey, "The cholinergic anti-inflammatory pathway: a missing link in neuroimmunomodulation," *Molecular medicine*, vol. 9, no. 5-8, pp. 125–134, 2003.

[50] M. Pohanka, "Butyrylcholinesterase as a biochemical marker," *Bratislava Medical Journal*, vol. 114, no. 12, pp. 726–734, 2013.

[51] L. Santarpia, I. Grandone, F. Contaldo, and F. Pasanisi, "Butyrylcholinesterase as a prognostic marker: a review of the literature," *Journal of Cachexia, Sarcopenia and Muscle*, vol. 4, no. 1, pp. 31–39, 2013.

[52] F. Meng, X. Yin, X. Ma, X.-D. Guo, B. Jin, and H. Li, "Assessment of the value of serum cholinesterase as a liver function test for cirrhotic patients," *Biomedical Reports*, vol. 1, no. 2, pp. 265–268, 2013.

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Research Article

Sepsis Induces a Dysregulated Neutrophil Phenotype That Is Associated with Increased Mortality

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Background. Neutrophil dysfunction in sepsis has been implicated in the pathogenesis of multiorgan failure; however, the role of neutrophil extracellular traps (NETs) remains uncertain. We aimed to determine the sequential changes in ex vivo NETosis and its relationship with mortality in patients with sepsis and severe sepsis. *Methods*. This was a prospective observational cohort study enrolling 21 healthy age-matched controls and 39 sepsis and 60 severe sepsis patients from acute admissions to two UK hospitals. Patients had sequential bloods for the ex vivo assessment of NETosis in response to phorbol-myristate acetate (PMA) using a fluorometric technique and chemotaxis using time-lapse video microscopy. Continuous data was tested for normality, with appropriate parametric and nonparametric tests, whilst categorical data was analysed using a chi-squared test. Correlations were performed using Spearman's rho. *Results*. Ex vivo NETosis was reduced in patients with severe sepsis, compared to patients with sepsis and controls ($p = 0.002$). PMA NETosis from patients with septic shock was reduced further ($p < 0.001$) compared to controls. The degree of metabolic acidosis correlated with reduced NETosis ($p < 0.001$), and this was replicated when neutrophils from healthy donors were incubated in acidotic media. Reduced NETosis at baseline was associated with an increased 30-day ($p = 0.002$) and 90-day mortality ($p = 0.014$) in sepsis patients. These findings were accompanied by defects in neutrophil migration and delayed apoptosis. Resolution of sepsis was not associated with the return to baseline levels of NETosis or migration. *Conclusions*. Sepsis induces significant changes in neutrophil function with the degree of dysfunction corresponding to the severity of the septic insult which persists beyond physiological recovery from sepsis. The changes induced lead to the failure to effectively contain and eliminate the invading pathogens and contribute to sepsis-induced immunosuppression. For the first time, we demonstrate that reduced ex vivo NETosis is associated with poorer outcomes from sepsis.

1. Introduction

The incidence of sepsis is continuing to rise and accounts for approximately 215,000 deaths per year in the United States of America (USA) [1]. The management of sepsis places a large financial burden on health care systems with conservative estimates suggesting that the USA spends $17 billion treating sepsis annually [1–3].

Sepsis is a complex syndrome that has been defined as a life-threatening immune response to infection [4]. However, the pathogen load and its virulence and the subsequent host characteristics determine the extent and nature of this response [2, 5]. Neutrophils are one of the first lines of defense against invading pathogens and are responsible for containing and eliminating invading pathogens [6, 7]. Neutrophils are multifaceted innate immune cells that also modulate the inflammatory response and initiate the adaptive immune responses to sepsis via the release of cytokines. It is this coordinated response that maintains immune homeostasis [8].

In sepsis, there is a dysregulated immune response with activated circulating neutrophils releasing cytokines and reactive oxygen species (ROS) at sites distal to the infectious

focus leading to multiorgan failure [7]. Additionally, neutrophils have been shown to demonstrate immunosuppressive phenotypes, with immaturity and altered chemokine expression responsible for some of these alterations [8]. This has pertinence in sepsis, with sepsis-induced immunosuppression being recognized as a clinical syndrome in survivors of sepsis, who have an increased susceptibility to nosocomial infections, frequent hospital readmissions, and subsequently increased late mortality [7, 9–11]. Recently, we described a reduction in systemic neutrophil migratory accuracy in lower respiratory tract infections, pneumonia, and a mild, ward-based pneumonia-associated sepsis cohort with evidence of prolonged migratory dysfunction after the septic event [12]. It is unclear whether reduced neutrophil migratory accuracy might also be a feature of a more severe sepsis cohort, how this might change over time, and whether other facets of neutrophil function might also be affected.

Following activation, triggered either by frustrated phagocytosis or sustained inflammation, neutrophils release neutrophil extracellular traps (NETs), whereby nuclear DNA laden with histones and granular contents are liberated into the extracellular space, which trap and kill extracellular bacteria [13, 14]. The exact role of NETs in sepsis remains uncertain. Studies in murine models of sepsis have shown that an inability to generate NETs in response to infection leads to increased severity of insult and death [14–17]. Additionally, similar studies have demonstrated that NET formation in intravascular beds, such as liver sinusoids, and within the alveoli causes endothelial damage and leads to patterns of organ dysfunction which are the hallmark of sepsis [18–22]. However, studies linking poor clinical outcomes with neutrophil functions, and the production of NETs in patients with sepsis over time, are lacking.

One of the hallmarks of sepsis is tissue hypoperfusion and tissue hypoxia leading to a switch to anaerobic glycolysis and the development of a metabolic lactic acidosis, with the degree of metabolic acidosis often used as a surrogate marker for severity of the inflammatory/infectious insult [4]. The role of acidosis in dysregulated neutrophil function in sepsis is poorly understood.

We hypothesized that sepsis severity (clinical and biochemical) would be associated with impairment of neutrophil functions, and in particular NETs, with worse clinical outcomes being seen in patients with most evidence of innate immunoparesis. Additionally, we hypothesized that the severity of acidosis would also be related to NET suppression. To test this, we aimed to assess neutrophil functions in patients hospitalized with sepsis and relate this to markers of sepsis severity and short and long-term clinical outcomes.

2. Materials and Methods

Adult patients admitted to a hospital with sepsis were screened and enrolled within 48 hours of admission to hospital. Sepsis, severe sepsis, and septic shock were defined based on the criteria used by the Surviving Sepsis Campaign Guidelines of 2008 (online Supplementary Materials available here) [23].

Patients were recruited from the University Hospital Birmingham and the Heart of England NHS Foundation Trust between September 2012 and June 2014 with 1-year follow-up completed in June 2015. Patients had blood drawn on enrollment and where possible on day 4 and day 7. Healthy aged controls (≥60 years with no systemic disease or only mild systemic disease; stage 1 hypertension/mild asthma) were also recruited.

This study was carried out per the Declaration of Helsinki, and all patients and healthy controls were consented. Patients were consented, and where not possible, assent was gained from their next of kin or physician. These studies received appropriate ethical approvals (Regional Ethics Committee references: 11/SC/0356 and 11/YH/0270). This research was undertaken prior to the publication of the new sepsis definitions of 2015. The new definitions identify a population at high risk of in-hospital mortality, but exclude milder infections, which this study sought to include [4].

2.1. Isolation of Neutrophils. Neutrophils were isolated on a Percoll (pH 8.5–9.5; Sigma-Aldrich, UK) density gradient as previously described [24, 25]. The neutrophils (95% pure and 97% viable by trypan blue exclusion) were resuspended in RPMI 1640 (Sigma-Aldrich).

2.2. Neutrophil Extracellular Trap Assay. Freshly isolated neutrophils (1×10^5 cells) were stimulated to generate NETs by incubating them within the control media (RPMI 1640 supplemented with glutamine, penicillin, and streptomycin-GPS; Sigma-Aldrich) or in the positive control 25 nM phorbol-myristate acetate (PMA, Sigma-Aldrich) as previously described using a fluorometric technique [26]. NET production was measured as arbitrary fluorescent units (AFUs).

2.3. Neutrophil Migration Assay. Migration was assessed using an Insall Chamber (Weber Scientific International Ltd., UK) as described previously [27, 28]. Migration was assessed towards a vehicle control (RPMI 1640), 100 nM interleukin-8 (CXCL-8) (R&D Systems, UK). Time-lapse video microscopy was used to capture neutrophil migration and analysed using ImageJ vector analysis software (Wayne Rasband, Bethesda) to calculate chemotaxis (directional migration) [12].

2.4. Cell-Free DNA Measurement. Cell-free DNA (cf-DNA) levels were measured from stored plasma samples using a fluorometric assay and SYTOX Green Dye (Life Technologies, UK) as previously described [29]. Values are represented as ng/ml of cf-DNA.

2.5. Neutrophil Apoptosis Assay. Freshly isolated neutrophils (1×10^5 cells) were suspended in RPMI 1640 supplemented with GPS. Apoptosis experiments were performed by flow cytometry ($CyaN_{ADP}$; Beckham Coulter) on isolation, at 4 hours and 24 hours.

To account for variations in baseline rates of apoptosis, the percentage change in apoptosis at 4 hours and 24 hours was calculated.

2.6. Statistical Analysis. Statistical analysis was performed using GraphPad Prism Version 6 (La Jolla, USA). Continuous data was tested for normality using a Shapiro-Wilk test.

Parametric data are represented as mean ± SEM and were analysed using a Student's *t*-test (two independent samples) or a one-way analysis of variance (ANOVA) test with a post hoc Bonferroni test (more than 2 groups). Nonparametric data are represented as median (IQR) and were analysed with a Mann–Whitney *U* test (two independent samples) or a Kruskal-Wallis test (more than 2 groups) with Dunn's post hoc test. A Pearson or Spearman correlation was used for parametric and nonparametric data, respectively. Categorical data was analysed using Fisher's exact test for two variables and a chi-squared (χ^2) test used when greater than two groups were analysed. All tests were two-tailed with results considered significant if $p < 0.05$.

3. Results

3.1. Participant Characteristics. 39 patients with sepsis, 60 patients with severe sepsis, and 21 age-matched healthy controls were recruited. Predictably, patients with sepsis had significantly greater comorbidities and were taking more medications than healthy controls were. However, the patients recruited into the sepsis and severe sepsis cohorts were well matched with no significant differences for age, sex, preexisting diseases, or medications being taken. The demographics of enrolled participants are shown in Table 1 with an experimental consort diagram shown in Figure 1.

3.2. Severe Sepsis, but Not Sepsis, Suppresses NET Production. Ex vivo NETosis in healthy controls were compared to patients diagnosed with sepsis and severe sepsis on admission to hospital. No differences were seen in baseline NETosis in unstimulated neutrophils between groups (8219 ± 2796 AFUs versus 7219 ± 4685 AFUs versus 7191 ± 5141 AFUs; ANOVA, $p = 0.701$). PMA-stimulated neutrophils from healthy controls and from patients with sepsis generate similar levels of NETs (healthy controls: 49659 ± 3285 AFUs versus sepsis: 45304 ± 1777 AFUs; Student's *t*-test, $p = 0.207$; Figure 2(a)). However, in neutrophils from patients with severe sepsis, NETosis (37181 ± 2204 AFUs) was significantly abrogated compared to both healthy controls (49659 ± 3285 AFUs) and patients with sepsis (45304 ± 1777 AFUs; ANOVA, $p = 0.002$; see Figure 2(a)). In a subgroup of patients with severe sepsis that had septic shock ($N = 13$), NETosis was further attenuated compared to the healthy controls (23785 ± 2853 AFUs versus 49,659 ± 3285 AFUs; Student's *t*-test, $p < 0.001$) and patients with sepsis (23785 ± 2853 AFUs versus 45304 ± 1777 AFUs, $p < 0.001$).

3.3. Persistent Attenuation of NETosis over Time. In patients with sepsis and severe sepsis, dynamic changes in NETosis were assessed on days 1, 4, and 7 where permitted (see Figure 2(b)). Patients with sepsis (day 1: 45304 ± 11096 AFUs versus day 4: 45081 ± 11047 versus day 7: 44871 ± 19480; ANOVA, $p = 0.99$) and severe sepsis (day 1: 37181 ± 2204 AFUs versus day 4: 37513 ± 1954 AFUs versus day 7: 40116 ± 2622 AFUs, ANOVA, $p = 0.68$) did not show any significant change in NETosis over time. On day 4, NETosis in patients with severe sepsis (37513 ± 1954 AFUs, $N = 39$) was significantly lower compared to sepsis patients at day 4 (45081 ± 2017 AFUs, $N = 30$) and healthy controls at baseline (ANOVA, $p = 0.001$; see Figure 2(b)). By day 7, despite most patients with severe sepsis showing signs of sepsis resolution (SOFA 0-1), NETosis was persistently reduced compared to healthy controls (40116 ± 2622 AFUs versus 49659 ± 3285 AFUs; Student's *t*-test, $p = 0.02$; see Figure 2(b)). Neutrophils from nonresolving donors of sepsis by day 7 (SOFA > 3, $N = 9$) showed a trend towards generating a lower number of NETs compared with resolvers and healthy aged controls (39347 ± 6103 versus 42545 ± 2502 versus 49659 ± 3285), but this failed to reach significance ($p = 0.17$, ANOVA). To detect a significant difference, with 80% power ($p = 0.05$), a total of 29 patients with nonresolving sepsis would be required.

3.4. Suppressed NETosis Is Associated with Increased Early and Late Mortality. Amongst all patients admitted with sepsis and severe sepsis, 16 died within 30 days of admission, with 80 surviving. Survivors of sepsis/severe sepsis had greater PMA-induced NETosis on admission than nonsurvivors had (47153 ± 1559 AFUs versus 34241 ± 4666 AFUs; Student's *t*-test $p = 0.002$; see Figure 3). By 90 days, mortality had risen to 24 patients and again was associated with an attenuated NETosis at admission to hospital (46772 ± 1744 versus 37498 ± 3602 AFUs; Student's *t* test, $p = 0.014$). There were no significant differences between survivors and nonsurvivors with regard to age at 30 days or 90 days.

The receiver operator curve (ROC) of survivors and nonsurvivors demonstrated an area under the curve of 0.70 (95% CI: 0.56–0.86; $p = 0.011$) with admission PMA-induced NET value of less than 39000 AFUs, having a 56% sensitivity and a 71% specificity for predicting 30-day mortality, whilst for 90-day mortality there was a 47% sensitivity and 70% specificity.

3.5. Suppression of NETosis Is Related to the Severity of Acidosis. The standardized base excess (SBE) is a measure of metabolic acidosis. Patients with severe sepsis had a lower average SBE than those with sepsis had, who had a lower average SBE than healthy controls. Patients with greater deficits in measured SBE, reflecting a more severe metabolic acidosis, demonstrated a reduction in NETosis (Spearman rho = 0.348, 95% CI 0.147–0.521, $p < 0.001$) (see Figure 4). We therefore hypothesized that the acidosis induced by sepsis may alter neutrophil functions and be causally associated with the reduction of NETosis seen in patients with severe sepsis.

To investigate this, neutrophils from healthy donors were incubated in media (RPMI 1640) at a range of pathophysiological pHs (pH 7.4, 7.2, and 7.0) for 40 minutes prior to NETosis experiments being carried out as described previously. As the pH of the control media was reduced, there was a sequential fall in healthy donor neutrophils' ability to generate NETs in response to PMA stimulation (see Figure 4), although significance was only seen when comparing a pH of 7.4 with 7.0. Viability assays performed demonstrated no significant alteration in neutrophil viability induced by changes in pH at 4 hours.

3.6. Cell-Free DNA Is Raised in Patients with Sepsis. Circulating levels of plasma cf-DNA were measured on patients

Table 1: Characteristics of healthy controls and patients with sepsis and severe sepsis enrolled in the study.

	Healthy controls	Sepsis	Severe sepsis	Septic shock	p value
N	21	39	60	13	
Age	70 (66–77)	75 (65–85)	73 (60–84)	71 (48–74)	0.519*
Sex, male (%)	13 (61.9)	20 (51.3)	41 (68.3)	10 (76.9)	0.070#
No. of comorbidities					
0	15	10	9	3	
1	6	17	21	5	<0.001Φ
2	0	7	19	3	
3+	0	5	11	2	
Comorbidities§, n					
None	15 (71)	10 (26)	9 (15)	3 (23)	
Hypertension	6 (29)	18 (46)	37 (62)	4 (31)	
IHD	0 (0)	10 (26)	15 (25)	2 (15)	
Asthma	0 (0)	0 (0)	0 (0)	1 (8)	0.076Φ
COPD	0 (0)	14 (36)	21 (35)	0 (0)	
Chronic renal disease	0 (0)	0 (0)	3 (5)	0 (0)	
Diabetes	0 (0)	3 (8)	17 (28)	4 (31)	
Medications§, n					
None	15	15	10	3	
Antihypertensive	6	6	28	2	
Beta-blocker	1	8	16	3	
Antiplatelet	0	13	16	2	<0.001Φ
Oral hypoglycemic	0	3	11	2	
Insulin	0	0	6	4	
Inhaled beta-agonist	0	6	16	0	
HMG-CoA reductase inhibitors	0	8	20	3	
APACHE II	N/A	14.5 (9–16)	16 (14–19)	19 (14–23)	<0.001
ICU Admission, N	N/A	0 (0%)	14 (23.3%)	13 (100%)	
Vasopressors alone			2	2	N/A
Respiratory support			4	3	
Multiorgan support			8	8	
WCC (10^9/l)		14.8 (12.3–19.0)	15.1 (12.6–19.8)	20.4 (10.2–26.3)	0.634**
CRP (mg/l)		153 (54.5–247)	100 (23.7–264)	132 (95–291)	0.232**
Lactate (nM)	N/A	1.7 (1.1–2.1)	2.5 (2.0–3.4)	2.6 (1.3–4.1)	<0.001**
SBE	N/A	0.2 (−1.2 to 2.6)	−2.2 (−5.6 to 0.5)	−4.0 (−7.2 to −2.2)	0.001**
SOFA score	N/A	1 (0-1)	3 (1–6)	9 (6–12)	<0.001**
Length of stay, days	N/A	10 (6–19)	10 (6.3–19.8)	21.5 (10–67)	0.505**
Mortality, n (%)					
30-day	0	4 (10)	12 (20)	7 (53)	0.265#
90-day	0	7 (18)	17 (28)	8 (62)	0.337#
360-day	0	11 (28)	22 (37)	8 (62)	0.513#

The baseline characteristics of healthy participants and patients recruited. The septic shock cohort is a subset of the patients with severe sepsis. The p values have been calculated using healthy, sepsis, and severe sepsis participant cohorts where appropriate. p values represented by * are from a Kruskal-Wallis test, # from a Fisher's exact test, Φ from a χ^2 test, and ** from a Mann–Whitney U test.

where sequential data points were available. Patients with sepsis (1308 ng/ml) and severe sepsis (1801 ng/ml) had significantly elevated levels of cf-DNA (ANOVA, $p < 0.001$) on admission compared to healthy controls (69 ng/ml) which persisted through to day 7 (ANOVA, $p < 0.001$) following admission (see online Supplementary Materials). No correlation was observed between NETosis and plasma cf-DNA ($p = 0.988$, Spearman's rho).

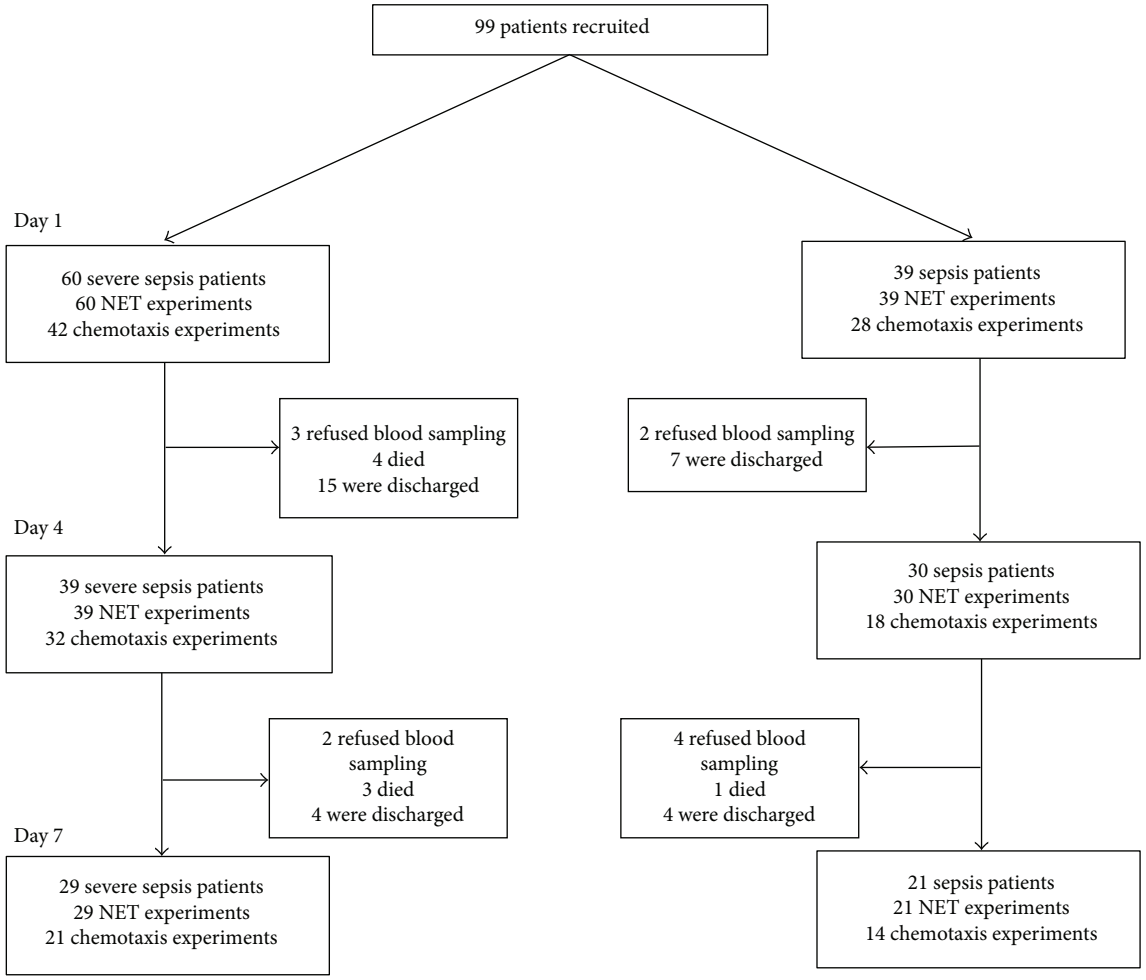

Figure 1: Consort diagram of experiments. Modified consort diagram of the number of experiments performed at the various time points during the study. The reducing number of experiments performed between the groups was due to either deaths, discharges from hospital, or refusals of blood sampling from patients.

3.7. Severe Sepsis Causes Aberrant Neutrophil Migration. Chemotaxis (directional migration) towards CXCl-8 on admission in patients with severe sepsis (0.15 μm/min, IQR 0.01–0.43 μm/min) was significantly reduced compared to patients with sepsis (0.49 μm/min, IQR 0.19–0.93 μm/min) and healthy age-matched donor neutrophils (0.86 μm/min {IQR 0.40–1.8 μm/min}, Kruskal-Wallis; $p < 0.001$). Although patients with sepsis (without organ dysfunction) showed reduced chemotaxis compared to healthy controls, this failed to reach statistical significance (Dunn's; $p = 0.15$). Dysfunctional chemotaxis in severe sepsis patients ($N = 32$) persisted through to day 4 (0.35 μm/min {IQR 0.09–0.85 μm/min} versus 0.86 μm/min {IQR 0.40–1.8 μm/min}; Dunn's; $p = 0.021$). By day 7, chemotaxis in severe sepsis patients ($N = 21$) remained below levels seen in healthy elderly donors (0.60 μm/min {IQR 0.30–1.1 μm/min} versus 0.86 μm/min {IQR 0.40–1.8 μm/min}), but this failed to reach significance (Dunn's; $p = 0.15$).

3.8. Apoptosis Is Delayed in Neutrophils from Sepsis Patients. Neutrophil apoptosis from 18 patients with severe sepsis and 19 age-matched healthy controls was measured at the time of neutrophil isolation, at 4 hours and 24 hours postisolation. Patients with severe sepsis patients had a greater number of neutrophils in early (18.8% {IQR 13–32%} versus 5.3% {IQR 3.9–6.7%} $p < 0.001$, Mann–Whitney U test) and late apoptosis (2.8% {IQR 1.0–3.6%} versus 1.2% {IQR 0.5–2.1%} $p = 0.03$, Mann–Whitney U test) compared to healthy controls at the time of isolation.

At 4 hours, neutrophils from severe sepsis patients showed no difference in early and late apoptosis compared to baseline (early: 19.6% {IQR 15–33%} versus 18.8% {IQR 12–32%}, $p = 0.19$; late: 2.8% {1.0 versus 3.3% versus 2.6% {1.3–4.0} $p = 0.45$, Wilcoxon signed-rank tests), whilst healthy neutrophils showed significant increased early apoptosis 4 hours following isolation (10.2% {IQR 8–12%) versus 5.3% {IQR 3.6–5.8%}, Wilcoxon signed-rank test; $p < 0.001$).

At 24 hours following isolation, the rates of early ($34.9 \pm 22\%$ versus $67.4 \pm 15\%$, $p < 0.001$) and late apoptosis ($9.4 \pm 2.3\%$ versus $19.8 \pm 1.7\%$, $p = 0.002$) were significantly lower in severe sepsis patients compared with healthy patients, suggesting that neutrophil survival is prolonged during sepsis.

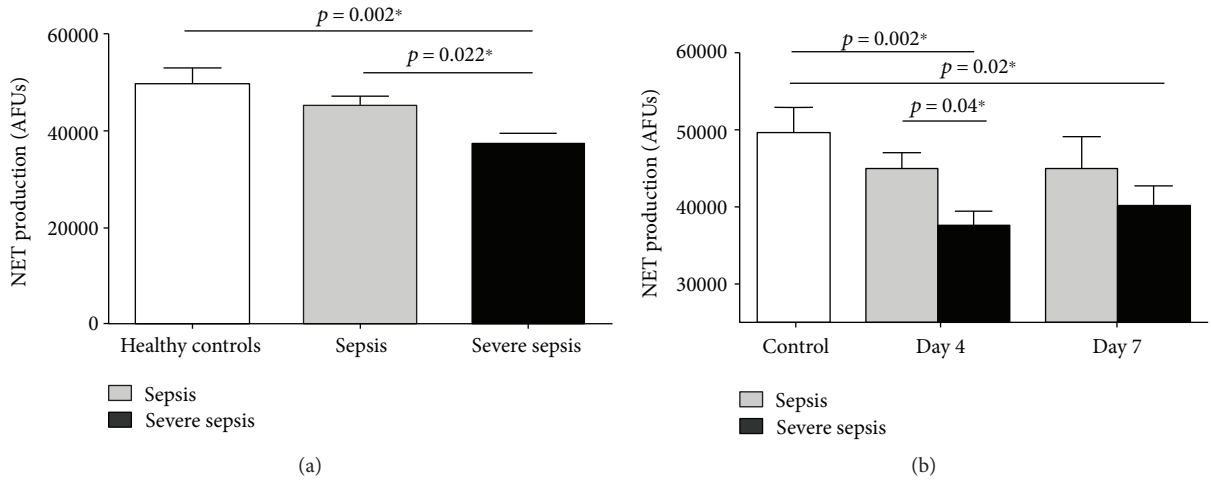

Figure 2: The ex vivo production of NETs in healthy controls and patients with sepsis and severe sepsis. (a) NETosis from 21 healthy controls, 39 patients with sepsis, and 60 patients with severe sepsis following stimulation with 25 nM PMA for 4 hours ex vivo. An ANOVA between all 3 groups showed a significant difference where $p = 0.002$, with $*$ representing the significant differences in a post hoc Tukey's test. (b) NETosis from 21 healthy controls, 30 patients with sepsis, and 39 patients with severe sepsis on day 4 following stimulation with 25 nM PMA for 4 hours ex vivo. An ANOVA between all 3 groups showed a significant difference ($p = 0.001$), with $*$ representing significant differences in a post hoc Tukey's test. On day 7, there are 21 sepsis patients and 29 severe sepsis patients. There was a significant difference between healthy controls and patients with severe sepsis**.

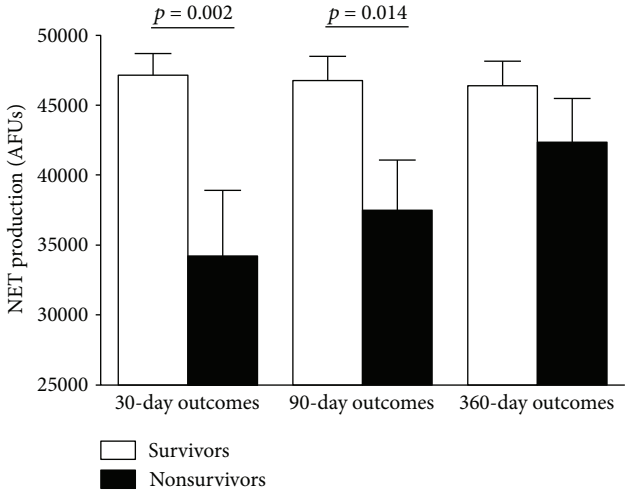

Figure 3: PMA-induced NET release is lower in nonsurvivors of sepsis at 30 days and 90 days. NET production in recruited patients on enrollment in response to PMA in survivors and nonsurvivors of sepsis at 30 days, 90 days, and 360 days following admission. Bars represent the mean with error bars the SEM, with p values from a Student's t-test.

4. Discussion

This study investigated the sequential changes in neutrophil functions including NETosis and migration in a large cohort of sepsis and severe sepsis patients and related this to clinically relevant outcomes. Confirming our initial hypothesis, we demonstrated that severe sepsis is associated with a reduction in NETosis in systemic neutrophils which is not present in milder forms of sepsis. Furthermore, in severe sepsis, reduced NETosis persists through to days 4 and 7. Reduced NETosis was associated with important clinical outcomes including short- (30-day) and medium-term (90-day) mortality. To our knowledge, this is the largest study of NETosis in sepsis and the first that reports a relationship with impairment in innate immune cell function and patient survival. Suppression of ex vivo NETosis below 39000 AFUs was performed comparatively with other traditional biomarkers in predicting mortality from sepsis such as the severity of acidosis (SBE) and lactate [30].

We also observed a correlation between the severity of acidosis and the attenuation of NETosis in patients with sepsis. Furthermore, by manipulating the pH of the cellular environment in vitro, we could recapitulate the septic neutrophil phenotype suggesting that the disruption of cellular acid-base homeostasis may contribute to dysfunctional NETosis and perhaps other neutrophil functions. Changes in extracellular pH are common in many inflammatory diseases and lead to neutrophil activation, phagocytosis, and ROS production [31, 32]. NET formation in relation to pH has not been studied before, and these data add to the literature of the negative impact of acid-base disturbance.

Our data suggest that sepsis and the associated inflammation and changes in blood pH may induce a neutrophil phenotype characterised by poor migratory accuracy, reduced NETosis, and prolonged neutrophil survival. Previously, we have described aberrant neutrophil migratory accuracy with sepsis in pneumonia, but the current study is the first to study neutrophil functions and survival in such a large cohort of sepsis patients [12].

NETosis is a terminal event for neutrophils; thus, neutrophil activation and predominance of antiapoptotic pathways induced with sepsis may inhibit NETosis [31–33]. However, NETosis studies in patients with sepsis are hindered due to the failure to capture early NETosis at sepsis onset. Our study describes no difference in baseline NET release but reduced NET release following activation in patients with severe sepsis

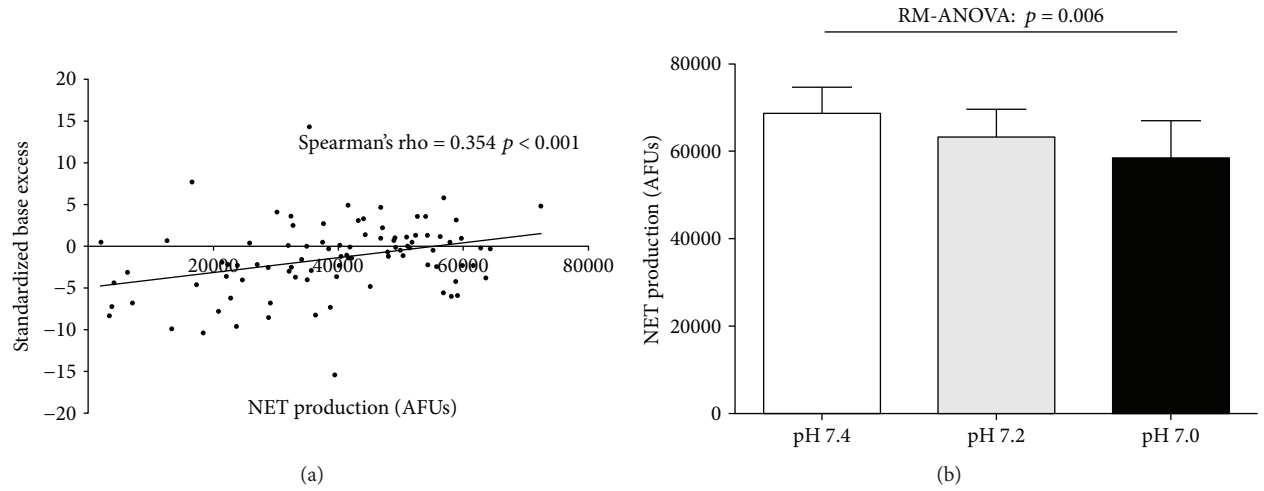

Figure 4: The impact of acidosis on neutrophil extracellular trap formation. (a) Correlation between standardized base excess and neutrophil extracellular trap formation in 99 patients with sepsis. Spearman's rho suggests that reduction in NET formation is associated with worsening acidosis. (b) Effect of acidosis on the NETs generated from the neutrophils of 5 healthy donors.

which does not recover at day 4 or 7 and a high burden of cf-DNA. These findings appear contradictory but are concordant with other studies of neutrophil function following a major host insult [34]. It has been proposed that the "first wave" of neutrophils to arrive at sites of infection is programmed for early NETosis in an attempt to contain the infection rapidly [35]. Those arriving later (which form the circulating pool on testing) may be activated by circulating cytokines and the septic environment and are resistant to apoptosis accounting for the attenuation of NETosis observed [32, 36].

The high levels of cell-free DNA would be consistent with evidence of an accumulation of NETs in inflammatory conditions caused by poor clearance [37]. Sepsis is associated with increased complement activity, and studies have shown that NETs activate the complement in vitro and deposited C1q inhibits NET degradation including a direct inhibition of DNase-I by C1q [38]. Clearance of nets may also be delayed due to suppression of DNases by renal/hepatic dysfunction present in multiorgan dysfunction [39]. This provides a potential mechanism for the presence of increased cell-free DNA with circulating neutrophils which are less able to undergo NETosis, as described in the current study and others [29, 40]. Cf-DNA is also gaining increasing attention, as a potential biomarker in sepsis, as it predicts outcomes from sepsis in patients admitted to intensive care and is relatively easy to measure [39, 41, 42]. However, more work is needed to determine the relationship of cf-DNA to cell functions and clinical outcomes.

The changes in neutrophil phenotype may drive further local and distant organ damage and predispose patients to secondary infections by inducing a state of immunosuppression [32, 43]. We propose a biphasic neutrophilic response to infection, with initial activation leading to migratory failure, frustrated phagocytosis, a release of large quantities of NETs, seen as cf-DNA, and a subsequent prolongation of neutrophil survival. The circulation of these free histones would activate the adaptive immune system, via dendritic cells, with the formation of anti-elastin, anti-histone, and anti-nuclear antibodies, as described in chronic inflammatory illnesses and autoimmune diseases, leading to further endothelial damage and exacerbating microcirculatory dysfunction [44, 45]. Sepsis-induced immunosuppression is also propagated by neutrophils that induce apoptosis in T-cells via the programmed death receptor and its ligand (PD-1/PD-L1) [8, 46, 47].

This study has limitations. Firstly, not all patients could have samples taken for analysis at all time points leading to the disparity in numbers across time points and assays. Patients with sepsis often experience a fluctuating course in their clinical recovery and require serial clinical blood tests, and missing time points are a common feature of studies of this nature [48, 49]. However, there were no differences in patient characteristics in all substudies to the main group, suggesting the data is representative of all patients.

Presepsis neutrophil function has not been assessed in these patients, and it is unclear whether preexisting neutrophil dysfunction is pathogenically associated with sepsis; it is possible that those with poorest outcomes had worse baseline neutrophil function. In survivors of the initial infectious insult, further neutrophil functions were not assessed beyond 7 days and so it is unclear if neutrophil function returns to levels expected in health following complete resolution or whether a permanent dysfunction results, predisposing these patients to further infectious insults.

A further limitation is the use of PMA as the stimulant to generate NETs. PMA is not physiological but causes maximal release of ROS and subsequently NETs by circumventing G receptor signaling [13]. This stimulus was chosen to assess maximal NET release, and similar concentrations have been used in several NET-related publications, enabling comparisons with published literature [13, 29, 40, 50].

5. Conclusions

Sepsis induces significant changes in neutrophil function. These may contribute to the failure of containment and the

dissemination of the infection, whilst exaggerating the dysregulated immune response that is the hallmark of sepsis [4]. We propose that the combined dysfunctions result in a phenotypic immunoparesed neutrophil that contributes to the high mortality in patients with sepsis. Finally, we propose that further investigation into NETosis and cf-DNA as potential future biomarkers to identify high-risk patients with sepsis is warranted.

Abbreviations

AFUs: Arbitrary fluorescent units
ANOVA: Analysis of variance
cf-DNA: Cell-free DNA
CXCL-8: Interleukin-8
GPS: Glutamine, penicillin, and streptomycin
IQR: Interquartile range
NET: Neutrophil extracellular trap
PD-1: Programmed death receptor-1
PMA: Phorbol-myristate acetate
ROS: Reactive oxygen species
SBE: Standardized base excess
SEM: Standard error of the mean
USA: United States of America.

Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

Ethical approval for the study was granted by the Yorkshire and Humber regional ethics committee (references: 11/SC/0356 and 11/YH/0270).

Consent

All patients and participants in this study provided informed consent. Where consent was not possible, assent from their next of kin or an appropriate professional consultee was sought. Retrospective consent was later confirmed where possible.

Disclosure

Jaimin M. Patel and Elizabeth Sapey are joint first authors.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Jaimin M. Patel, David R. Thickett, Fang Gao, and Elizabeth Sapey designed the study and undertook the analysis. Jaimin M. Patel, Dhruv Parekh, and Davinder Dosanjh undertook the patient recruitment, sample analysis, and laboratory work. Aaron Scott conducted the sample analysis. Jaimin M. Patel, Elizabeth Sapey, and David R. Thickett wrote the first draft. All authors have reviewed and approved the final version of paper.

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Supplementary Materials

Table 1: criteria used for the definition of patients with sepsis and severe sepsis. Table 2: cell-free DNA values in healthy controls and sepsis and severe sepsis patients at days 1 and 7. Figure 1: severe sepsis is associated with reduced chemotaxis. (A) The difference in chemotaxis between healthy aged controls, sepsis patients, and severe sepsis patients on admission to hospital. (B) Sequential changes in neutrophil chemotaxis in patients with sepsis (day 1 N=28, day 4 N=18, and day 7 N=14) and severe sepsis (day 1 N=42, day 4 N=32, and day 7 N=21). Bars represent the median and IQR with the error bars from Tukey's distribution. p values from a Kruskal-Wallis test. *(Supplementary Materials)*

References

[1] D. C. Angus, W. T. Linde-Zwirble, J. Lidicker, G. Clermont, J. Carcillo, and M. R. Pinsky, "Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care," *Critical Care Medicine*, vol. 29, no. 7, pp. 1303–1310, 2001.

[2] D. C. Angus and T. van der Poll, "Severe sepsis and septic shock," *The New England Journal of Medicine*, vol. 369, no. 9, pp. 840–851, 2013.

[3] N. K. J. Adhikari, R. A. Fowler, S. Bhagwanjee, and G. D. Rubenfeld, "Critical care and the global burden of critical illness in adults," *Lancet*, vol. 376, no. 9749, pp. 1339–1346, 2010.

[4] M. Singer, C. S. Deutschman, C. W. Seymour et al., "The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3)," *JAMA*, vol. 315, no. 8, pp. 801–810, 2016.

[5] R. S. Hotchkiss and I. E. Karl, "The pathophysiology and treatment of sepsis," *The New England Journal of Medicine*, vol. 348, no. 2, pp. 138–150, 2003.

[6] N. Borregaard, "Neutrophils, from marrow to microbes," *Immunity*, vol. 33, no. 5, pp. 657–670, 2010.

[7] K. A. Brown, S. D. Brain, J. D. Pearson, J. D. Edgeworth, S. M. Lewis, and D. F. Treacher, "Neutrophils in development of multiple organ failure in sepsis," *Lancet*, vol. 368, no. 9530, pp. 157–169, 2006.

[8] P. H. C. Leliefeld, C. M. Wessels, L. P. H. Leenen, L. Koenderman, and J. Pillay, "The role of neutrophils in immune dysfunction during severe inflammation," *Critical Care*, vol. 20, no. 1, p. 73, 2016.

[9] J. C. Alves-Filho, A. de Freitas, F. Spiller, F. O. Souto, and F. Q. Cunha, "The role of neutrophils in severe sepsis," *Shock*, vol. 30, Supplement 1, pp. 3–9, 2008.

[10] M. J. Delano, T. Thayer, S. Gabrilovich et al., "Sepsis induces early alterations in innate immunity that impact mortality to secondary infection," *Journal of Immunology*, vol. 186, no. 1, pp. 195–202, 2011.

[11] L. Fialkow, L. Fochesatto Filho, M. C. Bozzetti et al., "Neutrophil apoptosis: a marker of disease severity in sepsis and sepsis-induced acute respiratory distress syndrome," *Critical Care*, vol. 10, no. 6, p. R155, 2006.

[12] E. Sapey, J. M. Patel, H. L. Greenwood et al., "Pulmonary infections in the elderly lead to impaired neutrophil targeting, which is improved by simvastatin," *American Journal of Respiratory and Critical Care Medicine*, vol. 196, no. 10, pp. 1325–1336, 2017.

[13] V. Brinkmann, U. Reichard, C. Goosmann et al., "Neutrophil extracellular traps kill bacteria," *Science*, vol. 303, no. 5663, pp. 1532–1535, 2004.

[14] V. I. Landoni, P. Chiarella, D. Martire-Greco et al., "Tolerance to lipopolysaccharide promotes an enhanced neutrophil extracellular traps formation leading to a more efficient bacterial clearance in mice," *Clinical and Experimental Immunology*, vol. 168, no. 1, pp. 153–163, 2012.

[15] P. Li, M. Li, M. R. Lindberg, M. J. Kennett, N. Xiong, and Y. Wang, "PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps," *The Journal of Experimental Medicine*, vol. 207, no. 9, pp. 1853–1862, 2010.

[16] M. Arazna, M. P. Pruchniak, and U. Demkow, "Neutrophil extracellular traps in bacterial infections: strategies for escaping from killing," *Respiratory Physiology & Neurobiology*, vol. 187, no. 1, pp. 74–77, 2013.

[17] W. Meng, A. Paunel-Gorgulu, S. Flohe et al., "Depletion of neutrophil extracellular traps in vivo results in hypersusceptibility to polymicrobial sepsis in mice," *Critical Care*, vol. 16, no. 4, p. R137, 2012.

[18] T. Narasaraju, E. Yang, R. P. Samy et al., "Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis," *The American Journal of Pathology*, vol. 179, no. 1, pp. 199–210, 2011.

[19] S. R. Clark, A. C. Ma, S. A. Tavener et al., "Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood," *Nature Medicine*, vol. 13, no. 4, pp. 463–469, 2007.

[20] E. E. Gardiner and R. K. Andrews, "Neutrophil extracellular traps (NETs) and infection-related vascular dysfunction," *Blood Reviews*, vol. 26, no. 6, pp. 255–259, 2012.

[21] G. M. Thomas, C. Carbo, B. R. Curtis et al., "Extracellular DNA traps are associated with the pathogenesis of TRALI in humans and mice," *Blood*, vol. 119, no. 26, pp. 6335–6343, 2012.

[22] E. Villanueva, S. Yalavarthi, C. C. Berthier et al., "Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus," *Journal of Immunology*, vol. 187, no. 1, pp. 538–552, 2011.

[23] R. P. Dellinger, M. M. Levy, J. M. Carlet et al., "American Association of Critical-Care Nurses, American College of Chest Physicians, American College of Emergency Physicians., Canadian Critical Care Society., European Society of Clinical Microbiology and Infectious Diseases., European Society of Intensive Care Medicine., European Respiratory Society., International Sepsis Forum., Japanese Association for Acute Medicine., Japanese Society of Intensive Care Medicine., Society of Critical Care Medicine., Society of Hospital Medicine., Surgical Infection Society., World Federation of Societies of Intensive and Critical Care Medicine. Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2008," *Critical Care Medicine*, vol. 36, no. 1, pp. 296–327, 2008.

[24] S. K. Butcher, H. Chahal, L. Nayak et al., "Senescence in innate immune responses: reduced neutrophil phagocytic capacity and CD16 expression in elderly humans," *Journal of Leukocyte Biology*, vol. 70, no. 6, pp. 881–886, 2001.

[25] E. Sapey, H. Greenwood, G. Walton et al., "Phosphoinositide 3-kinase inhibition restores neutrophil accuracy in the elderly: toward targeted treatments for immunosenescence," *Blood*, vol. 123, no. 2, pp. 239–248, 2014.

[26] J. Hazeldine, P. Harris, I. L. Chapple et al., "Impaired neutrophil extracellular trap formation: a novel defect in the innate immune system of aged individuals," *Aging Cell*, vol. 13, no. 4, pp. 690–698, 2014.

[27] N. Andrew and R. H. Insall, "Chemotaxis in shallow gradients is mediated independently of PtdIns 3-kinase by biased choices between random protrusions," *Nature Cell Biology*, vol. 9, no. 2, pp. 193–200, 2007.

[28] E. Sapey, J. A. Stockley, H. Greenwood et al., "Behavioral and structural differences in migrating peripheral neutrophils from patients with chronic obstructive pulmonary disease," *American Journal of Respiratory and Critical Care Medicine*, vol. 183, no. 9, pp. 1176–1186, 2011.

[29] P. Hampson, R. J. Dinsdale, C. M. Wearn et al., "Neutrophil dysfunction, immature granulocytes, and cell-free DNA are early biomarkers of sepsis in burn-injured patients; a prospective observational cohort study," *Annals of Surgery*, vol. 265, no. 6, pp. 1241–1249, 2017.

[30] I. Smith, P. Kumar, S. Molloy et al., "Base excess and lactate as prognostic indicators for patients admitted to intensive care," *Intensive Care Medicine*, vol. 27, no. 1, pp. 74–83, 2001.

[31] A. S. Trevani, G. Andonegui, M. Giordano et al., "Extracellular acidification induces human neutrophil activation," *Journal of Immunology*, vol. 162, no. 8, pp. 4849–4857, 1999.

[32] F. E. Diaz, E. Dantas, M. Cabrera et al., "Fever-range hyperthermia improves the anti-apoptotic effect induced by low pH on human neutrophils promoting a proangiogenic profile," *Cell Death & Disease*, vol. 7, no. 10, article e2437, 2016.

[33] J. G. Wann, Y. H. Hsu, C. C. Yang et al., "Neutrophils in acidotic haemodialysed patients have lower intracellular pH and inflamed state," *Nephrology, Dialysis, Transplantation*, vol. 22, no. 9, pp. 2613–2622, 2007.

[34] J. Hazeldine, D. N. Naumann, E. Toman et al., "Prehospital immune responses and development of multiple organ dysfunction syndrome following traumatic injury: a prospective cohort study," *PLoS Medicine*, vol. 14, no. 7, article e1002338, 2017.

[35] B. G. Yipp and P. Kubes, "NETosis: how vital is it?," *Blood*, vol. 122, no. 16, pp. 2784–2794, 2013.

[36] G. Drifte, I. Dunn-Siegrist, P. Tissieres, and J. Pugin, "Innate immune functions of immature neutrophils in patients with sepsis and severe systemic inflammatory response syndrome," *Critical Care Medicine*, vol. 41, no. 3, pp. 820–832, 2013.

[37] A. Mahajan, M. Herrmann, and L. E. Munoz, "Clearance deficiency and cell death pathways: a model for the pathogenesis of SLE," *Frontiers in Immunology*, vol. 7, 2016.

[38] J. Leffler, M. Martin, B. Gullstrand et al., "Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease," *Journal of Immunology*, vol. 188, no. 7, pp. 3522–3531, 2012.

[39] K. Saukkonen, P. Lakkisto, V. Pettila et al., "Cell-free plasma DNA as a predictor of outcome in severe sepsis and septic shock," *Clinical Chemistry*, vol. 54, no. 6, pp. 1000–1007, 2008.

[40] M. Hashiba, A. Huq, A. Tomino et al., "Neutrophil extracellular traps in patients with sepsis," *The Journal of Surgical Research*, vol. 194, no. 1, pp. 248–254, 2015.

[41] R. Huttunen, T. Kuparinen, J. Jylhava et al., "Fatal outcome in bacteremia is characterized by high plasma cell free DNA concentration and apoptotic DNA fragmentation: a prospective cohort study," *PLoS One*, vol. 6, no. 7, article e21700, 2011.

[42] S. Margraf, T. Logters, J. Reipen, J. Altrichter, M. Scholz, and J. Windolf, "Neutrophil-derived circulating free DNA (cf-DNA/NETs): a potential prognostic marker for posttraumatic development of inflammatory second hit and sepsis," *Shock*, vol. 30, no. 4, pp. 352–358, 2008.

[43] J. K. Juss, D. House, A. Amour et al., "Acute respiratory distress syndrome neutrophils have a distinct phenotype and are resistant to phosphoinositide 3-kinase inhibition," *American Journal of Respiratory and Critical Care Medicine*, vol. 194, no. 8, pp. 961–973, 2016.

[44] A. P. Diamantopoulos, "Extracellular neutrophil traps: a novel therapeutic target in ANCA-associated vasculitis?," *Frontiers in Immunology*, vol. 4, 2013.

[45] K. Tillack, P. Breiden, R. Martin, and M. Sospedra, "T lymphocyte priming by neutrophil extracellular traps links innate and adaptive immune responses," *Journal of Immunology*, vol. 188, no. 7, pp. 3150–3159, 2012.

[46] J. Demaret, F. Venet, A. Friggeri et al., "Marked alterations of neutrophil functions during sepsis-induced immunosuppression," *Journal of Leukocyte Biology*, vol. 98, no. 6, pp. 1081–1090, 2015.

[47] A. C. Patera, A. M. Drewry, K. Chang, E. R. Beiter, D. Osborne, and R. S. Hotchkiss, "Frontline science: defects in immune function in patients with sepsis are associated with PD-1 or PD-L1 expression and can be restored by antibodies targeting PD-1 or PD-L1," *Journal of Leukocyte Biology*, vol. 100, no. 6, pp. 1239–1254, 2016.

[48] J. M. Patel, C. Snaith, D. R. Thickett et al., "Randomized double-blind placebo-controlled trial of 40 mg/day of atorvastatin in reducing the severity of sepsis in ward patients (ASEPSIS Trial)," *Critical Care*, vol. 16, no. 6, p. R231, 2012.

[49] T. R. Craig, M. J. Duffy, M. Shyamsundar et al., "A randomized clinical trial of hydroxymethylglutaryl-coenzyme a reductase inhibition for acute lung injury (the HARP Study)," *American Journal of Respiratory and Critical Care Medicine*, vol. 183, no. 5, pp. 620–626, 2011.

[50] V. Brinkmann, B. Laube, U. Abu Abed, C. Goosmann, and A. Zychlinsky, "Neutrophil extracellular traps: how to generate and visualize them," *Journal of Visualized Experiments*, no. 36, article e1724, 2010.

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Research Article

Selected Biomarkers Correlate with the Origin and Severity of Sepsis

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The microbial etiology and source of sepsis influence the inflammatory response. Therefore, the plasma levels of cytokines (IL-6, IL-8, and IL-10), chemokines (CCL2/MCP-1, MIP-1β), heparin-binding protein (HBP), soluble CD14 (sCD14), and cortisol were analyzed in blood from septic patients obtained during the first 96 hours of intensive care unit hospitalization. The etiology was established in 56 out of a total of 62 patients enrolled in the study. Plasma concentrations of MCP-1, sCD14, IL-6, and IL-10 were significantly higher in patients with community-acquired pneumonia (CAP; $n = 10$) and infective endocarditis (IE; $n = 11$) compared to those with bacterial meningitis (BM; $n = 18$). Next, cortisol levels were higher in IE patients than in those with BM and CAP, and at one time point, cortisol was also higher in patients with gram-negative sepsis when compared to those with gram-positive infections. Furthermore, cortisol and MCP-1 levels correlated positively with the daily measured SOFA score. In addition, HBP levels were significantly higher in patients with IE than in those with BM. Our findings suggest that MCP-1, sCD14, IL-6, IL-10, cortisol, and HBP are modulated by the source of sepsis and that elevated MCP-1 and cortisol plasma levels are associated with sepsis-induced organ dysfunction.

1. Introduction

Sepsis represents the main cause of death from infectious diseases. Current mainstays of sepsis therapy are early recognition and timely antibiotic administration [1]. Since the results of microbiology tests are usually significantly delayed and since they are negative in almost 40% of patients, the selection of the antibiotic therapy is usually empirical, based on the presumed source of the sepsis and the epidemiological situation (i.e., community- versus hospital-acquired infection) [2, 3]. This often results in the use of combinations of broad-spectrum antibiotics, which can lead to colonization by multiresistant flora and other side effects, including *Clostridium difficile* infection [4, 5]. Therefore, the prevention of the unnecessary use of broad-spectrum antibiotics and the promotion of the use of narrow-spectrum antibiotics are of paramount importance.

Blood culture remains the standard test for sepsis, although the results are not usually available until 24 to 48 hours after the collection. Thus, blood biomarkers may sometimes provide faster information for the assessment of the microbial etiology of the infection, thereby helping select the appropriate empiric antimicrobial therapy. A good example of a reliable biomarker is procalcitonin (PCT), which is intensively upregulated in gram-negative sepsis and community-acquired pneumonia (CAP) caused by

typical pathogens, mainly *Streptococcus pneumoniae* [6, 7]. Similarly, interleukin- (IL-) 6 in blood has been found in 10-fold higher concentrations in neonates with gram-negative sepsis than in children with gram-positive sepsis, and in adults with CAP due to typical bacterial pathogens in comparison to those with atypical or viral pneumonia [8, 9]. Although these data seem to be promising, the high concentrations of circulating biomarkers observed in septic patients were evaluated mostly for prognostic reasons, and only a limited number of studies analyzed their value for the etiologic diagnosis of sepsis [10, 11]. Furthermore, there is a lack of data about the influence of the source of sepsis on blood biomarkers that might be helpful in source control and in the selection of an appropriate antimicrobial therapy [12].

Therefore, we analyzed selected inflammatory parameters obtained from intensive care unit (ICU) patients with sepsis during the first four days (96 hours) of hospitalization and assessed their associations with the bacterial etiology and source of sepsis.

2. Patients and Methods

The study was approved by the local ethics committee (IRB00002721) and performed in accordance with the Convention on Human Rights and Biomedicine (Oviedo 1997). The study subjects were enrolled after signing the written informed consent. Patients not able to sign the consent were enrolled by two attending physicians; however, their specimens were analyzed only if they signed the consent after their recovery. A prospective cohort study was carried out between November 2010 and November 2014 in the ICU of the Department of Infectious, Tropical and Parasitic Diseases, Na Bulovce Hospital, Prague, Czech Republic.

The inclusion criteria were the standard diagnostic criteria for sepsis valid at the time of the beginning of the study [13]. The exclusion criteria consisted of severe immunodeficiency, including HIV infection, oncologic disease, transfer from another hospital, a life expectancy < 48 hours, and a duration of antimicrobial therapy > 24 hours prior to enrollment into the study. The severity of illness was assessed by the Acute Physiology and Chronic Health Evaluation (APACHE) II score using the worst clinical and laboratory values during the first 24 hours after admission, and the Sequential Organ Failure Assessment (SOFA) score was calculated daily for four consecutive days to evaluate organ dysfunction. The etiologic diagnosis was made by the detection of pathogenic bacteria in blood cultures, cerebrospinal fluid (CSF), or urine; by the detection of bacterial or viral DNA in clinical specimens; or by the positivity for specific acute antibodies in the case of Legionnaire's disease or influenza. The sources of sepsis were identified by CSF analysis, chest X-ray, esophageal echocardiography, abdominal ultrasonography, or CT scan of the chest and/or abdomen. Altogether, 62 septic patients were enrolled into the study. Their demographic and clinical data are presented in Table 1. For the specialized laboratory tests, the venous blood was collected into S-Monovette tubes with heparin (Sarstedt AG & Co., Nümbrecht, Germany). All blood samples were immediately centrifuged and stored at −80°C until further analysis.

Cytokines and chemokines, namely, IL-6, IL-8, IL-10, macrophage inflammatory protein- (MIP-) 1β, and monocyte chemoattractant protein- (MCP-) 1, were analyzed using FlowCytomix™ multiplex analysis (eBioscience, Bender MedSystems GmbH, Vienna, Austria) and six-color flow cytometry (BD FACSCanto™ II, BD Biosciences, CA, San Jose, USA) with FACSDiva™ software (BD). Next, commercial enzyme-linked immunosorbent assays (ELISA) were used to measure serum levels of soluble CD14 (R&D Systems, Minneapolis, MN, USA), and an in-house ELISA was used for the heparin-binding protein (HBP) analysis; the method for HBP detection is described elsewhere [14]. The analysis of cortisol was performed on an Architect i2000™ immunochemistry analyzer (Abbott, Chicago, IL, USA) using the fluorescence polarization immunoassay FPIA (Abbott).

Statistical analyses were performed using STATISTICA 12 Stat Soft. The data are presented as medians (quartiles or interquartile range). Levels that could not be detected were assigned values equal to the lower detection limit of the test. Differences between analyzed parameters in the groups were tested using the Mann–Whitney U test, and for comparisons between values within the etiology, $\alpha < 0.05$. Further, the Kruskal-Wallis test was used for multiple comparisons at the $\alpha < 0.05$ level. Spearman's correlation test was used for the determination of correlations between the variables. A certified biostatistician (Dipl.-Ing. Nikol Kaspříková) confirmed the use of the statistical methods.

3. Results

3.1. Patients. Out of the 62 enrolled subjects, 56 patients had a confirmed microbial etiology of sepsis, and 51 of them had confirmed either gram-positive or gram-negative etiology of sepsis. Figure 1 demonstrates the process of their selection. We analyzed data from 37 patients suffering from sepsis caused by gram-positive pathogens. The most frequent clinical diagnosis of the patients with gram-positive sepsis was bacterial meningitis (BM; $n = 12$), followed by infective endocarditis (IE; $n = 11$), CAP ($n = 5$), severe soft tissue infection (SSTI; $n = 3$), vertebral osteomyelitis ($n = 2$), biliary sepsis ($n = 2$), acute bacterial epiglottitis ($n = 1$), and sepsis of unknown source ($n = 1$). Additional 14 patients were diagnosed with sepsis due to gram-negative pathogens. The clinical diagnoses of these patients were urosepsis ($n = 6$) followed by biliary sepsis ($n = 2$), BM ($n = 2$), CAP ($n = 2$), meningococcal sepsis ($n = 1$), and acute enteritis ($n = 1$). The comparison of the baseline characteristics of the patients diagnosed with gram-positive and gram-negative sepsis is shown in Table 2. Next, for the evaluation of the influence of the source of sepsis, the data of patients diagnosed with BM were compared to the data of patients with IE and those with CAP. Baseline and etiological characteristics of these patient groups are presented in Tables 3 and 4. Initial total and system-related SOFA score values in the groups of patients evaluated based on source and etiology of sepsis are presented in Table 5.

Table 1: Demographic and clinical data of septic patients.

	$n = 62$	
Age in years, median (interquartile range)	61 (70–46)	
Gender (male/female)	30/32	
Site of infection	*n*	(%)
Bacterial meningitis	18	29.0
Infective endocarditis	11	17.7
Community-acquired pneumonia	10	16.1
Urinary tract infection	6	9.7
Severe soft tissue infection	6	9.7
Biliary sepsis	4	6.5
Vertebral osteomyelitis	2	3.2
Meningococcal sepsis	1	1.6
Acute bacterial epiglottitis	1	1.6
Acute enteritis	1	1.6
Diverticulitis	1	1.6
Unknown origin	1	1.6
Etiology	*n*	%
Gram-positive bacteria	37	59.7
Streptococcus pneumoniae	16	25.8
Methicillin-sensitive *Staphylococcus aureus*	9	14.5
Methicillin-resistant *Staphylococcus aureus*	3	4.8
Streptococcus agalactiae	3	4.8
Streptococcus pyogenes	2	3.2
Streptococcus viridans	1	1.6
Streptococcus mitis	1	1.6
Streptococcus anginosus	1	1.6
Listeria monocytogenes	1	1.6
Gram-negative bacteria	14	22.6
Escherichia coli	5	8.1
Klebsiella pneumoniae	2	3.2
Pseudomonas aeruginosa	2	3.2
Neisseria meningitidis	2	3.2
Haemophilus influenzae non B	1	1.6
Legionella pneumophila	1	1.6
Salmonella group B	1	1.6
Polymicrobial infection	3	4.8
Methicillin-sensitive *Staphylococcus aureus*, *Peptostreptococcus* spp., *Bacteroides fragilis*	1	1.6
Fusobacterium nucleatum, *Porphyromonas* spp.	1	1.6
Citrobacter freundi, *Klebsiella oxytoca*	1	1.6
Virus	2	3.2
Influenza A H1N1	2	3.2
Pathogen not identified	6	9.7

3.2. Detection of Inflammatory Mediators in the Specimens. Altogether, we collected 1948 specimens, and the selected analytes were detected in 1687 (86.6%) samples. The plasma levels of MCP-1, sCD14, and HBP were detectable in all specimens collected during the study period. The plasma levels of cortisol and MIP-1β were detectable in most specimens, followed by the levels of IL-8, IL-6, and IL-10. The exact data are presented in Table 6.

3.3. Inflammatory Parameters in Gram-Positive and Gram-Negative Sepsis. Comparison of the biomarkers between patients with gram-positive and gram-negative sepsis demonstrated a very limited number of significant differences. Notably, plasma levels of cortisol were significantly higher in patients with gram-negative sepsis than in those with gram-positive sepsis only on day 2 of the study. Although the MCP-1 concentration demonstrated a similar trend in patients with gram-negative infections over the whole study period, it did not reach statistical significance. The median concentrations of the examined biomarkers and their statistical comparisons are presented in Table 7.

3.4. Inflammatory Parameters and the Source of Sepsis. The plasma levels of MCP-1, sCD14, cortisol, IL-6, IL-10, and HBP were significantly decreased in patients with BM in comparison to those with either IE or CAP. A significant difference between patients with IE and CAP was found in cortisol plasma levels only during one time point, namely, on day 4. Notably, patients with BM in comparison to those with CAP and IE had significantly decreased plasma levels of MCP-1 at all time points and sCD14 on days 2, 3, and 4. The level of sCD14 on day 1 was lower in patients with BM compared to those with CAP. The plasma cortisol levels were lower in patients with BM in comparison to those with IE on days 2, 3, and 4 and on day 3 in comparison to those with CAP. Similar findings in patients with BM were also demonstrated for IL-10 in comparison to those with CAP on days 1, 3, and 4 and in comparison to those with IE on days 1 and 2. Patients with BM also had significantly lower IL-6 plasma levels in comparison to those of patients with CAP on days 2 and 3. Additionally, the level of HBP was significantly lower in patients with BM when compared to those with IE on days 1 and 3. The biomarker concentrations in the evaluated sources of sepsis and their statistical comparisons are presented in Table 8.

3.5. Correlations among the Laboratory Parameters and the SOFA Score. We assessed the correlation among the SOFA score and the inflammatory parameters with a detectability above 90%. Two of the selected sepsis biomarkers—MCP-1 and cortisol—demonstrated consistently significant correlations with the daily SOFA score over the study period. These data are presented in Table 9.

4. Discussion

In the current study, we observed significant relationships between plasma levels of MCP-1, sCD14, HBP, and cortisol and the source of sepsis, whereas the well-established inflammatory cytokines (i.e., IL-6 and IL-10) demonstrated relatively low detectability in our cohort of septic patients. Furthermore, plasma MCP-1 and cortisol levels were significantly correlated with the SOFA score, and cortisol was also correlated with the gram-negative etiology of sepsis.

Blood concentrations of chemokines had already been evaluated with a cytometric bead array in a study that

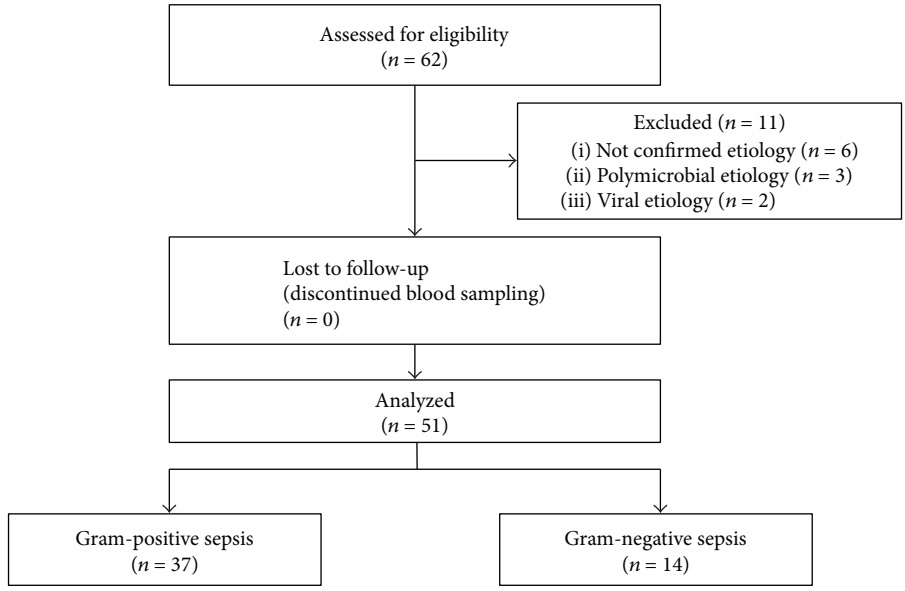

Figure 1: Flow chart of the patient selection process for the analysis based on confirmed gram-positive and gram-negative etiology of sepsis.

Table 2: The comparison of baseline characteristics of patients with gram-positive ($n = 37$) and gram-negative ($n = 14$) sepsis.

	Gram positive $n = 37$	Gram negative $n = 14$	P
Age, years	59 (66–42)	69.5 (75–35)	0.485
Gender (male/female)	19/18	6/8	—
APACHE II	16 (21–13)	21 (24–14)	0.261
SOFA	6 (5–8)	7.5 (6–9)	0.182
WBC (cells/μL)	16,400 (24,600–10,250)	14,750 (29,000–9100)	0.883
CRP (mg/L)	263 (319–179)	181 (267–111)	**0.026**

Data are expressed as medians (interquartile range). APACHE II: Acute Physiology and Chronic Health Evaluation II; SOFA: Sequential Organ Failure Assessment; WBC: white blood cell count; CRP: C-reactive protein.

Table 3: The comparison of baseline characteristics of patients with bacterial meningitis ($n = 18$), infective endocarditis ($n = 11$), and community-acquired pneumonia ($n = 10$).

	Bacterial meningitis $n = 18$	Infective endocarditis $n = 11$	Community-acquired pneumonia $n = 10$	P
Age, years	64 (69-70)	59.5 (32–66)	48 (33–62)	0.140
Gender (male/female)	7/11	5/6	6/4	
APACHE II	18.5 (16–21)	10.5 (8–17)	21.5 (23.25–13.75)	0.038
SOFA	6.5 (5–8)	5.5 (2–7.5)	7 (5–8)	0.109
WBC (cells/μL)	22,000 (15,000–29,500)	11,250 (9400–20,550)	9600 (15,825–6050)	0.410
CRP (mg/L)	187 (119–312)	282 (243–313)	288 (414–107.5)	0.972

Data are expressed as medians (interquartile range). APACHE II: Acute Physiology and Chronic Health Evaluation II; SOFA: Sequential Organ Failure Assessment; WBC: white blood cell count; CRP: C-reactive protein.

enrolled 89 children with acute bacterial infections, which included patients with CAP, sepsis, and bacterial abscesses [15]. In that study, the median plasma MCP-1 levels in all three cohorts were significantly lower than those observed in 20 healthy controls (i.e., 24.9 pg/mL), which authors explained by a relatively long duration of the illness, with a median of six days before enrollment in the study. In any case, these concentrations were low. In another study, an analysis of baseline cytokine concentrations with a Luminex™ assay demonstrated serum MCP-1 levels of 62.8 pg/mL in healthy males and 55.4 pg/mL in healthy females [16]. Regarding IE and CAP, our findings are in agreement with those of a study performed in 137 patients with CAP that demonstrated mean plasma MCP-1 levels of 803.2 pg/

Table 4: The etiology of bacterial meningitis ($n = 18$), infective endocarditis ($n = 11$), and community-acquired pneumonia ($n = 10$).

	n (%)
Bacterial meningitis	18
Streptococcus pneumoniae	11 (61)
Streptococcus agalactiae	1 (5.6)
Listeria monocytogenes	1 (5.6)
Neisseria meningitidis	1 (5.6)
Fusobacterium nucleatum, *Porphyromonas* spp.	1 (5.6)
Haemophilus influenzae non B	1 (5.6)
Pathogen not identified	2 (11)
Infective endocarditis	11
Staphylococcus aureus (methicillin sensitive)	7 (63.6)
Staphylococcus aureus (methicillin resistant)	1 (9.1)
Streptococcus pyogenes	1 (9.1)
Streptococcus mitis	1 (9.1)
Streptococcus viridans	1 (9.1)
Community-acquired pneumonia	10
Streptococcus pneumoniae	5 (50)
Legionella pneumoniae	1 (10)
Escherichia coli	1 (10)
Influenza A	2 (20)
Pathogen not identified	1 (10)

mL after admission [17]. The variability of the concentrations found in different studies can be explained by the specific cohorts of patients and the use of different analytic methods, that is, Luminex, ELISA, or cytometric arrays. Additionally, a blood collection method can be responsible for the differences among detected concentrations, because in some studies plasma was used and in others serum was used. On the other hand, in our previous study in which serum concentrations of MCP-1 were detected with the Luminex method in 21 adult patients with bacterial infection, almost the same concentrations were found compared to the current study using plasma and the cytometric assay method [18]. This may suggest that the source of sepsis is a more important factor than the analytic method used for MCP-1 detection. In addition, the positive correlation of the plasma MCP-1 levels with the SOFA score in all 62 enrolled patients may indicate the importance of this chemokine in sepsis outcome. This has already been described in children with meningococcal sepsis whose serum MCP-1 levels correlated positively ($r = 0.68$) with their SOFA score and in adults who died of sepsis and who had significantly higher serum MCP-1 levels than the levels in survivors [19, 20]. Moreover, MCP-1 plasma levels have been extensively studied in gram-negative infections. High levels were found in experimental endotoxemia in baboons after sublethal and lethal doses of *Escherichia coli* [21]. As in this experimental study, elevated plasma levels of MCP-1 were found in humans with sepsis and septic shock; however, no differences were found between patients with gram-positive and gram-negative bacterial infections [22]. In contrast to these findings, we observed a trend for higher plasma MCP-1 levels in patients with gram-negative sepsis compared to the levels in those with gram-positive sepsis. Nevertheless, our observation is in line with findings in meningococcal sepsis or urosepsis—important gram-negative infections—that are associated with high MCP-1 concentrations in blood [23, 24].

Cortisol is an important sepsis biomarker. Elevated cortisol concentrations have been described in septic shock and gram-positive sepsis [25]. In contrast to this finding, we observed the highest cortisol levels in patients with gram-negative sepsis. This may be partly explained by a smaller proportion of BM patients (14%) in the gram-negative group compared to 37% of patients with BM in the gram-positive group, because the lowest cortisol levels in our study were found in BM patients. It is worth noting that the highest cortisol levels (>3000 nmol/mL) were observed in a patient with gram-negative sepsis arising from the biliary tract, in a patient with *E. coli* pneumonia complicated with venous thromboembolism (VTE), and in a patient with fulminant meningococcal sepsis who survived with severe sequelae (data not shown). Higher plasma cortisol levels have already been demonstrated in survivors of meningococcal sepsis in comparison to nonsurvivors, and in patients with Cushing's syndrome with increased risk of VTE; however, there have been no studies of cortisol levels in patients with biliary sepsis [26, 27]. It seems probable that these findings represent the severity of those clinical situations. Next, it has also been suggested that decreased plasma cortisol levels are not rare in septic shock patients and that poor outcomes cannot be predicted from cortisol levels in blood per se but rather by adrenocortical hyporesponsiveness [28]. Despite this paradigm, we observed a positive correlation between plasma cortisol levels and the daily SOFA score over the whole study period. Higher SOFA scores were found in septic patients with relative adrenal insufficiency when compared to patients with severe courses of sepsis without this alteration, but a direct association between the SOFA score and plasma cortisol levels was not reported [29]. It is well known that elevated cortisol levels may reflect the normal response of the hypothalamic-pituitary-adrenal (HPA) axis in sepsis, which is important for catecholamine effects and vasopressin release. Additionally, cortisol is responsible for maintaining the vascular response to catecholamines, leading to the maintenance of an adequate blood perfusion pressure of vital organs [30]. Interestingly, the elevation of cortisol levels in patients with IE is, to our knowledge, an original finding. Since the major difference was observed between patients with IE and those with BM, we can assume that the plasma cortisol levels were modulated by corticosteroid therapy, which is routinely administered to BM patients based on generally accepted guidelines and avoided in IE patients because of its relative contraindication [31]. In addition, we observed significantly higher cortisol levels in patients with IE than in those with CAP, which may also indicate an intense activation of the HPA axis during IE. Specific host reactions in IE are also supported by the observation of elevated plasma HBP levels at two time points in the study. It should be stressed that HBP may cause vascular injury and leakage in sepsis, and it may be possible that HBP plays the same role in bloodstream infections [32].

Table 5: Total and system-related SOFA score values in the groups of patients evaluated based on source and etiology of sepsis.

System	SOFA score				
	Bacterial meningitis $n = 18$	Infective endocarditis $n = 11$	Community-acquired pneumonia $n = 10$	Gram-positive sepsis $n = 37$	Gram-negative sepsis $n = 14$
Respiration	1.5 (0–2)	0 (0–2)	3 (2-3)	1 (0–2)	2 (0–2)
Coagulation	0 (0-1)	1 (0–3)	0 (0-1)	1 (0-1)	0 (0–2)
Liver	0 (0–0)	0 (0-1)	0 (0-1)	0 (0-1)	0 (0–2)
Cardiovascular	0.5 (0–2)	0 (0–2)	2 (1-2)	0 (0–2)	2 (0–2)
Central nervous system	3 (3–3)	0 (0–2)	0 (0-1)	1 (0–3)	0 (0–2)
Renal	0 (0-1)	1 (0-1)	2 (0–4)	0 (0-1)	1.5 (0–2)
SOFA total	6.5 (5–8)	5.5 (2–7.5)	7 (5–8)	6 (5–8)	7.5 (6–9)

Data are presented as medians (interquartile range). SOFA: Sequential Organ Failure Assessment.

Table 6: Detectability of laboratory markers during the study.

Parameter	Day 1 Detected/total		Day 2 Detected/total		Day 3 Detected/total		Day 4 Detected/total		Total (4 days) Detected/total	
MIP-1β	57/61	93.4%	58/62	93.5%	56/62	90.3%	58/62	93.5%	229/247	92.7%
MCP-1	61/61	100%	62/62	100%	62/62	100%	62/62	100%	247/247	100%
IL-10	36/61	59.0%	28/62	45.2%	27/62	43.5%	24/62	38.7%	115/247	46.6%
IL-8	54/61	88.5%	53/62	85.5%	52/62	83.9%	54/62	87.1%	213/247	86.2%
IL-6	50/61	82.0%	46/62	67.7%	42/62	67.7%	37/62	59.7%	175/247	70.9%
HBP	57/57	100%	59/59	100%	59/59	100%	59/59	100%	234/234	100%
sCD14	61/61	100%	62/62	100%	62/62	100%	62/62	100%	247/247	100%
Cortisol	56/56	100%	57/58	98.3%	57/59	96.6%	57/59	96.6%	227/232	97.8%

Increased serum levels of sCD14 have already been described in 54 patients with gram-negative septic shock with a median concentration of 3.23 mg/L, which was significantly higher than the median concentration in healthy controls (2.48 mg/L). In that study, using the ELISA method for sCD14 detection, the highest concentrations were observed in nonsurvivors in comparison to survivors—4.2 versus 2.8 mg/L, respectively [33]. Moreover, another study demonstrated that neonates with gram-negative sepsis had significantly higher sCD14 levels in their blood than did children with gram-positive sepsis [34]. We observed a similar trend in patients with gram-negative sepsis; however, it did not reach statistical significance. Additionally, we did not find a correlation between the severity of sepsis and the concentration of circulating sCD14. Similarly, no differences were found between the plasma sCD14 concentrations of survivors and nonsurvivors in a cohort of 142 adult patients with serious community-acquired infections [35]. Furthermore, not only the etiology but also the source of sepsis can influence the blood levels of sCD14. However, to our knowledge, there is a limited number of studies comparing plasma sCD14 levels in patients with different sources of sepsis arising from community-acquired infections. In our previous study aimed at identifying potential biomarkers of community-acquired bacterial and viral infections, no differences were found in serum sCD14 concentrations between patients with CAP and those with urosepsis [36]. The levels of sCD14 observed in the current study in patients with CAP seem to be higher than those detected in the previous study (4.50 versus 3.71 mg/L). This difference can reflect either the severity of disease, as the previous study was executed in the standard wards only, or the etiology of CAP, since some patients had mild-to-moderate pneumonia caused by atypical pathogens. Additionally, a blood type specimen could have played a role, because we compared levels in plasma and serum. Next, some studies reported similar sCD14 concentrations in patients with BM as those observed in the current study. For example, in one study, children with meningococcal meningitis and sepsis had a median plasma sCD14 concentration of 3.3 mg/L, which is only slightly higher than the levels found in our patients with BM [37]. The blood levels of sCD14 were also evaluated as a prognostic factor of CAP, and very high blood levels with a median concentration of 6.07 mg/mL were found in 198 survivors in comparison to the 6.70 mg/mL median level observed in nonsurvivors [38]. Since no information about the etiology of CAP was given in this study and a different scoring system was used for the assessment of the severity of pneumonia, it is impossible to compare these data with those of our study. We cannot compare our findings in patients with IE with those of other studies because, to our knowledge, there have been no studies performed about sCD14 levels in patients with IE. In addition, recent studies have frequently utilized a fragment of sCD14 denoted as sCD14-ST, also known as presepsin, suggesting that this glycoprotein is an interesting diagnostic sepsis biomarker [39].

Table 7: The comparison of biomarker levels between gram-positive and gram-negative sepsis.

Parameter	Day 1		Day 2		Day 3		Day 4		P
	G+	G−	G+	G−	G+	G−	G+	G−	
MCP-1 (pg/mL)	576.40 (1188.61)	685.84 (4291.12)	505.33 (692.46)	786.23 (1593.18)	450.84 (742.37)	715.29 (848.93)	415.96 (438.42)	745.97 (616.07)	n.s.
MIP-1β (pg/mL)	43.49 (62.26)	48.99 (75.74)	43.03 (79.45)	39.39 (67.09)	39.11 (72.34)	28.45 (65.29)	37.78 (86.18)	36.79 (47.61)	n.s.
IL-6 (pg/mL)	47.74 (133.21)	18.96 (399.91)	20.98 (56.49)	27.98 (202.13)	11.28 (40.93)	11.21 (295.56)	2.65 (40.24)	15.02 (41.50)	n.s.
IL-8 (pg/mL)	80.54 (192.72)	106.62 (150.94)	60.14 (187.78)	31.43 (89.09)	65.83 (174.18)	40.56 (107.72)	63.23 (208.04)	34.49 (98.74)	n.s.
IL-10 (pg/mL)	6.32 (15.18)	1.90 (47.42)	1.90 (8.84)	3.64 (6.48)	1.90 (12.34)	1.90 (15.91)	1.90 (8.44)	1.90 (3.94)	n.s.
HBP (ng/mL)	236.33 (462.47)	288.59 (894.53)	122.42 (267.37)	162.18 (532.89)	111.85 (238.57)	124.47 (232.94)	82.94 (113.00)	115.96 (388.1)	n.s.
sCD14 (mg/L)	3.62 (1.34)	3.89 (1.12)	3.59 (1.48)	4.11 (1.14)	3.58 (1.39)	3.82 (1.24)	3.68 (2.24)	3.28 (1.64)	n.s.
Cortisol (nmol/L)	326.90 (290.20)	442.80 (400.25)	396.20* (421.4)	662.00* (2038.60)	423.20 (431.20)	545.55 (538.2)	452.60 (335.5)	416.15 (381.50)	**0.048**

Data are presented as median (quartile); n.s.: not significant; *$P < 0.05$: gram-positive (G+) versus gram-negative (G−) infection. The statistical difference-analyzed parameters in the groups were analyzed by the Mann–Whitney U test.

Table 8: The comparison of biomarker levels among the groups based on the source of sepsis.

Parameter	Day 1			Day 2			Day 3			Day 4		
	BM	IE	CAP	BM	IE	CAP	BM	IE	CAP	BM	IE	CAP
MCP-1 (pg/mL)	133.8[1,2] (149.0)	542.4[1] (693.2)	685.6[2] (1064.9)	130.0[1,2] (158.5)	624.5[1] (454.3)	667.7[2] (509.3)	154.1[1,2] (136.6)	489.5[1] (274.4)	581.5[2] (513.5)	221.1[1,2] (216.9)	471.8[1] (334.4)	605.3[2] (378.9)
MIP-1β (pg/mL)	25.0 (67.0)	40.6 (56.7)	23.1 (73.4)	14.2 (63.7)	29.7 (74.8)	12.8 (36.2)	16.4 (45.8)	30.1 (82.4)	13.8 (22.5)	23.3 (73.9)	26.9 (90.9)	13.4 (26.4)
IL-6 (pg/mL)	12.8 (46.5)	22.1 (113.8)	49.9 (191.9)	1.6[1,2] (10.4)	29.9[1] (76.3)	18.4[2] (91.2)	1.2[1,2] (6.9)	29.9[1] (61.0)	8.1[2] (96.0)	1.2 (12.1)	42.5 (92.1)	1.2 (25.9)
IL-8 (pg/mL)	13.1 (133.7)	37.3 (157.4)	47.1 (102.9)	14.2 (169.6)	34.7 (126.8)	22.6 (43.5)	32.3 (151.1)	33.6 (191.1)	34.4 (99.5)	44.7 (130.9)	52.4 (185.5)	25.1 (34.0)
IL-10 (pg/mL)	1.9[1,2] (0.0)	11.9[1] (17.7)	10.2[2] (6.6)	1.9[1] (0.0)	9.8[1] (14.4)	1.9 (7.6)	1.9[1] (0.0)	5.2[1] (14.5)	9.5 (13.3)	1.9[2] (0.0)	1.9 (7.2)	12.5[2] (14.2)
HBP (ng/mL)	110.1[1] (225.1)	369.6[1] (826.3)	96.7 (187.0)	92.1 (165.4)	189.5 (362.6)	57.2 (140.4)	66.8 (134.5)	230.2 (457.0)	102.5 (130.8)	43.4 (77.1)	111.3 (224.8)	155.2 (332.8)
sCD14 (mg/L)	3.0[2] (1.0)	4.0 (1.3)	4.5[2] (0.6)	2.8[1,2] (0.6)	4.2[1] (1.2)	4.0[2] (1.1)	2.6[1,2] (0.5)	3.9[1] (0.8)	3.7[2] (2.1)	2.3[1,2] (1.5)	4.0[1] (1.6)	4.2[2] (2.9)
Cortisol (nmol/L)	174.3 (146.9)	556.2 (531.9)	324.7 (555.8)	95.1[1] (75.7)	509.5[1] (450.8)	290.6 (377.4)	76.7[1,2] (88.7)	528.7[1] (217.8)	215.1[2] (329.3)	111.3[1] (248.5)	531.5[1,3] (143.3)	220.6[3] (305.6)

Data are presented as median (quartile); BM: bacterial meningitis; IE: infective endocarditis; CAP: community-acquired pneumonia; [1]$P < 0.05$: BM versus IE; [2]$P < 0.05$: BM versus CAP; [3]$P < 0.05$: IE versus CAP. The statistical difference-analyzed parameters for multiple comparison were analyzed by the Kruskall-Wallis test.

Table 9: The relationship of biomarker levels and SOFA score during the study.

Parameter	SOFA Day 1	SOFA Day 2	SOFA Day 3	SOFA Day 4
MCP-1 (pg/mL)	0.425*	0.510*	0.355*	0.440*
Cortisol (nmol/L)	0.336*	0.382*	0.499*	0.415*
sCD14 (mg/L)	0.061	0.074	0.118	0.237*
MIP-1β (pg/mL)	0.039	0.107	0.081	0.085
HBP (ng/mL)	−0.032	−0.021	−0.108	−0.140

*$P < 0.05$. Spearman's correlation test was used for determination of correlations. SOFA: Sequential Organ Failure Assessment.

Inflammatory cytokines have been extensively studied in sepsis. They have multiple functions during severe infections and sepsis, and some of them are considered reliable biomarkers that are useful for daily practice, especially in ICU patients. A good example is IL-6, the blood levels of which should correlate with the severity and bacterial etiology of sepsis [36, 40]. Based on these findings, IL-6 measurement has become routine in many clinical laboratories. However, in this study we observed an unacceptable IL-6 detectability, reaching approximately 82% on day 1 with a subsequent decrease to approximately 68% on days 2 and 3 to finally 60% on the last day of the study. A study with 70 septic shock patients demonstrated 64% detectability of IL-6 in sera at the study entry [41]. These data are relatively close to our finding on day 2 of the study, and they indicate the diagnostic limitation of IL-6 levels in septic patients. Another potential biomarker of sepsis is IL-8, which has strong chemoattractant and activating effects on neutrophils. Elevated plasma levels of this cytokine were found in septic patients, with a median concentration of 280 pg/mL, and IL-8 was detectable with the ELISA method in 89% of all examined specimens [42]. This detectability is very close to that found in our study, in which an average detectability of 86.2% was demonstrated over the study period. Sepsis is also associated with an intense anti-inflammatory response initiated almost simultaneously with the initial proinflammatory reactions. Therefore, IL-10, one of the strongest anti-inflammatory cytokines, can be detected in the blood during the acute phase of sepsis. In a study with 69 septic patients, the plasma levels of IL-10 were detectable with the ELISA method in heparinized blood in 39 (57%) of the enrolled subjects; the median IL-10 concentration was higher in patients with septic shock (58 pg/mL) in comparison to those with sepsis (11 pg/mL) [43]. If we compare our data with results of that study, we observed a lower detectability of IL-10, with a value of 46.6% compared to the 57% in the abovementioned study. Possible explanations of this difference could be that we did not examine specimens from patients with fatal outcomes or that we used the multiplex cytometric method for cytokine and chemokine detection. In any case, IL-10 is a less reliable biomarker than any other investigated inflammatory mediator in our study.

Our study has certain limitations. First, this is a single-center study from a highly specialized ICU for infectious disease patients, which is reflected in the relatively high proportions of specific infectious diseases—BM and IE. On the other hand, this also represents an advantage because of the high proportion of patients with established etiological diagnoses. Second, the patients were enrolled in the study the morning after their admission to the ICU; specimens collected immediately upon admission were only obtained from 30 subjects and therefore were not evaluated. Consequently, we did not assess the influence of antimicrobial therapy on the analyzed biomarkers. Third, we could not assess mortality and examine specimens from patients with fatal outcome, because the local ethics committee requested informed consent from relatives of succumbed patients, which we felt traumatic for their surviving family. Therefore, important data including mortality rate and differences in selected biomarkers among survivors and nonsurvivors are missing. Lastly, the groups of patients with evaluated infectious diseases and cohorts of patients with gram-positive and gram-negative infections were relatively small, and the primary aim of the study was not to test a diagnostic value of the selected biomarkers.

5. Conclusion

Altogether, our data indicate that MCP-1 and cortisol are promising biomarkers with a potential for differentiation between gram-positive and gram-negative sepsis and for evaluation of severity of the clinical course of sepsis. Moreover, it is apparent that blood levels of MCP-1, cortisol, sCD14, and HBP are significantly modulated by the source of sepsis, suggesting their pathophysiological and diagnostic importance, which should be further tested in a large cohort of septic patients.

Data Availability

Requests for data, 12 months after initial publication, will be considered by the corresponding author.

Conflicts of Interest

None of the authors have declared direct financial relations with the trademarks mentioned in the paper that might lead to a conflict of interests.

Authors' Contributions

Michal Holub designed and coordinated the study, supervised the data analysis, and drafted the manuscript; Olga Džupová carried out the clinical part of the study and analyzed the data; Michaela Růžková analyzed the data and helped in drafting the manuscript; Alžběta Stráníková carried out the cytometric and ELISA analysis and participated in the data analysis; Eva Bartáková helped with the data analysis and in the presentation of the results; Jan Máca helped with the data interpretation and in drafting the manuscript; Jiří Beneš enrolled some patients; Heiko Herwald contributed to the study design, performed the HBP analysis, and helped in drafting the manuscript; and Ondřej Beran contributed to the study design and the presentation of the data and helped in drafting the manuscript.

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References

[1] A. Rhodes, L. E. Evans, W. Alhazzani et al., "Surviving sepsis campaign: international guidelines for management of sepsis and septic shock: 2016," *Intensive Care Medicine*, vol. 43, no. 3, pp. 304–377, 2017.

[2] B. A. Cunha, "Empiric antimicrobial therapy for bacteremia: get it right from the start or get a call from infectious disease," *Clinical Infectious Diseases*, vol. 39, no. 8, pp. 1170–1173, 2004.

[3] J. Phua, W. Ngerng, K. See et al., "Characteristics and outcomes of culture-negative versus culture-positive severe sepsis," *Critical Care*, vol. 17, no. 5, article R202, 2013.

[4] E. Meyer, F. Schwab, B. Schroeren-Boersch, and P. Gastmeier, "Dramatic increase of third-generation cephalosporin-resistant *E. coli* in German intensive care units: secular trends in antibiotic drug use and bacterial resistance, 2001 to 2008," *Critical Care*, vol. 14, no. 3, article R113, 2010.

[5] J. D. Chalmers, A. R. Akram, A. Singanayagam, M. H. Wilcox, and A. T. Hill, "Risk factors for *Clostridium difficile* infection in hospitalized patients with community-acquired pneumonia," *Journal of Infection*, vol. 73, no. 1, pp. 45–53, 2016.

[6] S. Li, H. Rong, Q. Guo, Y. Chen, G. Zhang, and J. Yang, "Serum procalcitonin levels distinguish gram-negative bacterial sepsis from gram-positive bacterial and fungal sepsis," *Journal of Research in Medical Sciences*, vol. 21, no. 1, p. 39, 2016.

[7] J. Hedlund and L. O. Hansson, "Procalcitonin and C-reactive protein levels in community-acquired pneumonia: correlation with etiology and prognosis," *Infection*, vol. 28, no. 2, pp. 68–73, 2000.

[8] I. H. Celik, G. Demirel, N. Uras, E. S. Oguz, O. Erdeve, and U. Dilmen, "The role of serum interleukin-6 and C-reactive protein levels for differentiating aetiology of neonatal sepsis," *Archivos Latino-Americanos de Pediatría*, vol. 113, no. 6, pp. 534–537, 2015.

[9] K. Zobel, P. Martus, M. W. Pletz et al., "Interleukin 6, lipopolysaccharide-binding protein and interleukin 10 in the prediction of risk and etiologic patterns in patients with community-acquired pneumonia: results from the German competence network CAPNETZ," *BMC Pulmonary Medicine*, vol. 12, no. 1, 2012.

[10] F. A. Bozza, J. I. Salluh, A. M. Japiassu et al., "Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis," *Critical Care*, vol. 11, no. 2, article R49, 2007.

[11] K. Reinhart, M. Bauer, N. C. Riedemann, and C. S. Hartog, "New approaches to sepsis: molecular diagnostics and biomarkers," *Clinical Microbiology Reviews*, vol. 25, no. 4, pp. 609–634, 2012.

[12] J. Rello, F. Valenzuela-Sánchez, M. Ruiz-Rodriguez, and S. Moyano, "Sepsis: a review of advances in management," *Advances in Therapy*, vol. 34, no. 11, pp. 2393–2411, 2017.

[13] M. M. Levy, M. P. Fink, J. C. Marshall et al., "2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference," *Intensive Care Medicine*, vol. 29, no. 4, pp. 530–538, 2003.

[14] H. Tapper, A. Karlsson, M. Mörgelin, H. Flodgaard, and H. Herwald, "Secretion of heparin-binding protein from human neutrophils is determined by its localization in azurophilic granules and secretory vesicles," *Blood*, vol. 99, no. 5, pp. 1785–1793, 2002.

[15] N. V. Cavalcanti, L. C. Torres, M. C. da Matta et al., "Chemokine patterns in children with acute bacterial infections," *Scandinavian Journal of Immunology*, vol. 84, no. 6, pp. 338–343, 2016.

[16] A. Biancotto, A. Wank, S. Perl et al., "Baseline levels and temporal stability of 27 multiplexed serum cytokine concentrations in healthy subjects," *PLoS One*, vol. 8, no. 12, article e76091, 2013.

[17] K. K. Yong, J. H. Chang, M. H. Chien et al., "Plasma monocyte chemoattractant protein-1 level as a predictor of the severity of community-acquired pneumonia," *International Journal of Molecular Sciences*, vol. 17, no. 2, 2016.

[18] M. Holub, D. A. Lawrence, N. Andersen et al., "Cytokines and chemokines as biomarkers of community-acquired bacterial infection," *Mediators of Inflammation*, vol. 2013, Article ID 190145, 7 pages, 2013.

[19] C. L. Vermont, J. A. Hazelzet, E. D. de Kleijn, G. P. van den Dobbelsteen, and R. de Groot, "CC and CXC chemokine levels in children with meningococcal sepsis accurately predict mortality and disease severity," *Critical Care*, vol. 10, no. 1, article R33, 2006.

[20] T. H. Hong, C. H. Chang, W. J. Ko et al., "Biomarkers of early sepsis may be correlated with outcome," *Journal of Translational Medicine*, vol. 12, no. 1, p. 146, 2014.

[21] P. M. Jansen, J. van Damme, W. Put, I. W. de Jong, F. B. Taylor, and C. E. Hack, "Monocyte chemotactic protein 1 is released during lethal and sublethal bacteremia in baboons," *The Journal of Infectious Diseases*, vol. 171, no. 6, pp. 1640–1642, 1995.

[22] A. W. Bossink, L. Paemen, P. M. Jansen, C. E. Hack, L. G. Thijs, and J. Van Damme, "Plasma levels of the chemokines monocyte chemotactic proteins-1 and -2 are elevated in human sepsis," *Blood*, vol. 86, no. 10, pp. 3841–3847, 1995.

[23] A. S. W. Møller, A. Bjerre, B. Brusletto, G. B. Joø, P. Brandtzaeg, and P. Kierulf, "Chemokine patterns in meningococcal disease," *Journal of Infectious Diseases*, vol. 191, no. 5, pp. 768–775, 2005.

[24] D. P. Olszyna, J. M. Prins, P. E. P. Dekkers et al., "Sequential measurements of chemokines in urosepsis and experimental endotoxemia," *Journal of Clinical Immunology*, vol. 19, no. 6, pp. 399–405, 1999.

[25] R. M. H. Schein, C. L. Sprung, E. Marcial, L. Napolitano, and B. Chernow, "Plasma cortisol levels in patients with septic shock," *Critical Care Medicine*, vol. 18, no. 3, pp. 259–263, 1990.

[26] K. F. M. Joosten, E. D. de Kleijn, M. Westerterp et al., "Endocrine and metabolic responses in children with meningoccocal sepsis: striking differences between survivors and nonsurvivors," *The Journal of Clinical Endocrinology & Metabolism*, vol. 85, no. 10, pp. 3746–3753, 2000.

[27] M. Zilio, L. Mazzai, M. T. Sartori et al., "A venous thromboembolism risk assessment model for patients with Cushing's syndrome," *Endocrine*, vol. 52, no. 2, pp. 322–332, 2016.

[28] J. L. Moran, M. J. Chapman, M. S. O'Fathartaigh, A. R. Peisach, P. R. Pannall, and P. Leppard, "Hypocortisolaemia and adrenocortical responsiveness at onset of septic shock," *Intensive Care Medicine*, vol. 20, no. 7, pp. 489–495, 1994.

[29] Y. S. Kwon, G. Y. Suh, E. H. Kang et al., "Basal serum cortisol levels are not predictive of response to corticotropin but have prognostic significance in patients with septic shock," *Journal of Korean Medical Science*, vol. 22, no. 3, pp. 470–475, 2007.

[30] V. Gheorghiță, A. E. Barbu, M. L. Gheorghiu, and F. A. Căruntu, "Endocrine dysfunction in sepsis: a beneficial or deleterious host response?," *Germs*, vol. 5, no. 1, pp. 17–25, 2015.

[31] F. McGill, R. S. Heyderman, S. Panagiotou, A. R. Tunkel, and T. Solomon, "Acute bacterial meningitis in adults," *The Lancet*, vol. 388, no. 10063, pp. 3036–3047, 2016.

[32] P. Bentzer, J. Fisher, H. J. Kong et al., "Heparin-binding protein is important for vascular leak in sepsis," *Intensive Care Medicine Experimental*, vol. 4, no. 1, p. 33, 2016.

[33] R. Landmann, W. Zimmerli, S. Sansano et al., "Increased circulating soluble CD14 is associated with high mortality in gram-negative septic shock," *The Journal of Infectious Diseases*, vol. 171, no. 3, pp. 639–644, 1995.

[34] A. Blanco, G. Solis, E. Arranz, G. D. Coto, A. Ramos, and J. Telleria, "Serum levels of CD14 in neonatal sepsis by gram-positive and gram-negative bacteria," *Acta Paediatrica*, vol. 85, no. 6, pp. 728–732, 1996.

[35] H. Aalto, A. Takala, H. Kautiainen, S. Siitonen, and H. Repo, "Monocyte CD14 and soluble CD14 in predicting mortality of patients with severe community acquired infection," *Scandinavian Journal of Infectious Diseases*, vol. 39, no. 6-7, pp. 596–603, 2009.

[36] P. Chalupa, O. Beran, H. Herwald, N. Kaspříková, and M. Holub, "Evaluation of potential biomarkers for the discrimination of bacterial and viral infections," *Infection*, vol. 39, no. 5, pp. 411–417, 2011.

[37] E. Arranz, A. Blanco-Quiroös, P. Solís, and J. A. Garrote, "Lack of correlation between soluble CD14 and IL-6 in meningococcal septic shock," *Pediatric Allergy and Immunology*, vol. 8, no. 4, pp. 194–199, 1997.

[38] A. Tejera, F. Santolaria, M. L. Diez et al., "Prognosis of community acquired pneumonia (CAP): value of triggering receptor expressed on myeloid cells-1 (TREM-1) and other mediators of the inflammatory response," *Cytokine*, vol. 38, no. 3, pp. 117–123, 2007.

[39] F. F. Larsen and J. A. Petersen, "Novel biomarkers for sepsis: a narrative review," *European Journal of Internal Medicine*, vol. 45, pp. 46–50, 2017.

[40] C. E. Hack, E. R. De Groot, R. J. Felt-Bersma et al., "Increased plasma levels of interleukin-6 in sepsis," *Blood*, vol. 74, no. 5, pp. 1704–1710, 1989.

[41] T. Calandra, J. Gerain, D. Heumann, J. D. Baumgartner, M. P. Glauser, and The Swiss-Dutch J5 immunoglobulin study group, "High circulating levels of interleukin-6 in patients with septic shock: evolution during sepsis, prognostic value, and interplay with other cytokines," *The American Journal of Medicine*, vol. 91, no. 1, pp. 23–29, 1991.

[42] C. E. Hack, M. Hart, R. J. van Schijndel et al., "Interleukin-8 in sepsis: relation to shock and inflammatory mediators," *Infection and Immunity*, vol. 60, no. 7, pp. 2835–2842, 1992.

[43] A. Marchant, J. Devière, B. Byl, D. De Groote, J. L. Vincent, and M. Goldman, "Interleukin-10 production during septicaemia," *The Lancet*, vol. 343, no. 8899, pp. 707-708, 1994.

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Research Article

Significant Cytokine mRNA Expression Changes Immediately after Initiation of Cardiopulmonary Resuscitation

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Introduction. The purpose of this study was to evaluate immediate immunological changes following cardiopulmonary resuscitation (CPR). mRNA expression levels of selected immunomodulatory cytokines in out-of-hospital cardiac arrest (OHCA) survivors were detected and correlated to clinical parameter. *Methods*. OHCA survivors with sustained unconsciousness after return of spontaneous circulation (ROSC) were included. PAXgene whole blood samples were drawn immediately after initiation of CPR and subsequently after 6 h, 12 h, 24 h, 48 h, and 72 h. TNF-alpha, IL-8, IL-10, and IL-1ra mRNA levels were quantified by RT-qPCR and compared to multiple organ failure, 30-day survival, and the induction of therapeutic hypothermia (TH). *Results*. 25 patients (63 ± 15 years) were enrolled presenting a characteristic time-dependent cytokine profile in the early postresuscitation period. High initial TNF-alpha and IL-8 mRNA levels were followed by a significant decrease. IL-1ra mRNA levels significantly increased beginning after 6 h. Nonsurvivors showed significantly higher IL-8 mRNA levels immediately after CPR. TH induced significantly higher IL-1ra mRNA levels compared to normothermia. *Conclusion*. Significant mRNA cytokine expression changes are already detectable immediately after initiation of CPR. These expressional changes are significantly different depending on 30-day survival. TH seems to attenuate proinflammatory immune reaction by a significant increase of IL-1ra mRNA levels. This trial is registered with DRKS00012940.

1. Introduction

Survival of out-of-hospital cardiac arrest (OHCA) is affected by global ischaemia followed by a pronounced reperfusion injury due to return of spontaneous circulation (ROSC). The ischaemia-reperfusion response induces a complex activation of immunologic pathways leading to subsequent systemic inflammatory immune reaction. This phenomenon is known as the postcardiac arrest syndrome (PCAS). The underlying pathophysiological changes are known to be characterized by a fundamental dysregulation of both, pro- and anti-inflammatory cytokines, including IL-6, IL-8, TNF-alpha, IL-1ra, and IL-10 [1]. The duration of whole-body ischaemia and the subsequent inflammatory release have been identified to influence the severity of PCAS [2, 3]. Furthermore, the extent of these cytokine disorders seems to be associated with postresuscitation outcome [4]. To our knowledge, the majority of clinical studies evaluating cytokine changes started blood sampling when patients arrived at the hospital or were admitted to the ICU. One major interest of this study was to already detect very early expressional cytokine changes. Therefore, blood samples were drawn immediately after initiation of CPR in the preclinical setting in order to elucidate very early functional changes of the human immune system via investigating mRNA expression profiles of selected immunomodulatory

genes directly following cardiac arrest. This study further correlated cytokine mRNA expression changes with clinical outcome parameters such as multiple organ failure and 30-day mortality. Secondly, we evaluated the impact of therapeutic hypothermia on cytokine expression profiles as therapeutic hypothermia (TH) has—on the one hand—been shown to beneficially influence mortality rates after cardiac arrest (CA) [5–12]. However, data concerning the impact of hypothermia on the postresuscitation inflammation and the underlying mechanisms of PCAS-related cytokine changes is still conflicting [13–15].

2. Methods

2.1. Study Setting and Population. Patients (≥18 years) successfully resuscitated from OHCA with sustained unconsciousness (GCS < 8) after ROSC were included between January 2003 and March 2006 after approval of Regional Ethical Review Board of the Ludwig-Maximilians University, Munich, Germany (decision: 282/01). Written informed consent was obtained from each patient's next of kin and/or from patients regaining consciousness after cardiac arrest. The study was performed in accordance with the Declaration of Helsinki and its amendments and European Union guidelines for good clinical practice. Main exclusion criteria were acute infection, intracerebral hemorrhage, aortic dissection, or pregnancy. Prehospital data regarding the cardiac arrest including initial heart rhythm, witnessed collapse, bystander CPR, and time to ROSC were collected according to Utstein guidelines [16, 17]. Further, clinical parameters and comorbidities, cause of cardiac arrest, TH treatment, presence of cardiogenic shock, use of intra-aortic balloon pump (IABP), and the duration of stay in the intensive care unit (ICU) and in the hospital were recorded for each patient. This study was designed to (1) analyze very early mRNA expression profiles of TNF-alpha, IL-8, IL-10, and IL-1ra beginning already after CPR and within the first 72 h after successful cardiopulmonary resuscitation, (2) correlate cytokine levels with clinical outcome parameters, and (3) evaluate the effect of TH on cytokine mRNA expression levels. *Multiple organ failure* (*MOF*) was detected using MOF score as described by Goris et al. and modified by Lefering et al. [18, 19]. A MOF score ≥ 4 was surmised as multiple organ failure. If clinical data sets were incomplete, patients were excluded from this subanalysis. *Survival* was defined as 30-day survival after cardiac arrest. Patients who did not survive the first 24 h after resuscitation were excluded. Therapeutic hypothermia (TH) was not yet implemented in Resuscitation Guidelines at the time of sample collection and therefore not induced regularly. Our patients were treated in eight different hospitals, and a standardized TH protocol was therefore unavailable. In order to reduce heterogeneity, patients were retrospectively divided into two subgroups. Only patients treated according to the same TH protocol were included into the hypothermia group. Hypothermia was achieved by external cooling methods (blanket, ice packs) and associated with routine use of neuromuscular blocking agents. Targeted levels of hypothermia were 32–34°C and maintained for 24 h.

2.2. Patient Management. Survivors of OHCA were admitted to one of the eight intensive care units in Munich. All patients received intensive care treatment according to the current guidelines including mechanical ventilation, fluid substitution, antibiotic therapy, SIRS/sepsis management, and vasopressor treatment. Patients were sedated and received adequate analgesia according to the standard of each ICU. Percutaneous coronary intervention was performed if necessary.

2.3. Blood Sampling. Blood samples were drawn immediately after initiation of cardiopulmonary resuscitation by one of our ambulance emergency physicians (central ambulance station/LMU Munich) and subsequently 6, 12, 24, 48, and 72 h by one of the study's investigators.

2.4. RNA Extraction. Blood was collected in PAXgene tubes, and total RNA was extracted using the *PAXgene Blood RNA Kit* (PreAnalytix, Hombrechtikon, Swiss) according to the manufacturer's instructions. RNA quantity was measured with Nanodrop spectrophotometer ND-1000 (Thermo Scientific).

2.5. cDNA Synthesis. An aliquot of 8.2 μl standardized to 1 μg of RNA was reverse transcribed with avian myeloblastosis virus—reverse transcriptase (AMV-RT) and oligo-p(dT)15-primer—following manufacturer's instruction (1st Strand cDNA Synthesis Kit, Roche, Mannheim, Germany). After denaturation (Thermocycler, 65°C for 15 min), samples were iced for 5 minutes and incubated with manufacture's master-mix (11.8 μl including MgCl2, deoxynucleotide-mix, oligo-p(dT)15-primer, AMV reverse transcriptase, and RNase inhibitor).

2.6. Quantitative Real-Time PCR (RT-qPCR). Quantitative real-time PCR was performed in this study. The obtained cDNA was diluted 1 : 25 with water and 10 μl were used for amplification. The quantitative analysis of target gene expression was performed on a LightCycler by real-time PCR using the Light Cycler Fast Start DNA Master Sybr Green I Kit according to the manufacturer's instructions (Roche, Mannheim, Germany). Standard and primer were designed by *Search LC* (Heidelberg, Germany; Light Cycler Primer Sets); RT-qPCR was performed by denaturation and polymerase activation (95°C for 10 min), amplification of RT-qPCR products in a 45 cycle one-step PCR including denaturation (95°C, 10 s)/annealing (68°C–58°C, −0.5°C/cycle)/extension (72°C, 16 s) for each circle. The results of negative control samples were set as baseline level. Results of RT-qPCR were standardized to this baseline level and given as copies/μlRNA. Specificity of the amplification products was verified by melting curve analysis combined with agarose gel electrophoresis. For calculation, data processing and documentation of the LightCycler analysis software version 3.39 (Roche Diagnostics, Germany) was used.

2.7. Statistical Analysis. Data were statistically analyzed using ANOVA analysis (*Kruskal-Wallis*) and post hoc Dunn's Test in order to evaluate significant differences regarding the time points (0 h (A), 6 h, 12 h, 24 h, 48 h, and 72 h). Subgroups

Table 1: Patient characteristics.

Pt.	Gender (m/f)	Age (year)	Diagnosis Initial heart rhythm	Number of shocks	Hypothermia	Outcome
1	m	62	Respiratory failure (COPD) Asystole	—	U	S
2	m	76	Myocardial infarction Ventricular fibrillation	6	U	S
3	m	63	Respiratory failure (COPD) Ventricular fibrillation	1	U	NS
4	m	32	Asphyxia Asystole	—	U	NS
5	m	34	Chest trauma Asystole	—	U	S
6	m	61	Myocardial infarction Asystole	—	U	S
7	m	86	Myocardial infarction Asystole	—	U	NS
8	m	56	Myocardial infarction Ventricular fibrillation	3	U	S
9	m	45	Myocardial infarction Ventricular fibrillation	3	U	S
10	m	44	Myocardial infarction Ventricular fibrillation	5	U	S
11	m	67	Myocardial infarction Asystole	—	U	S
12	f	72	Lung embolism Asystole	—	U	NS
13	m	54	Unknown Ventricular fibrillation	3	N	NS
14	m	61	Myocardial infarction Ventricular fibrillation	6	N	S
15	m	90	Cardiac arrhythmia Ventricular fibrillation	1	N	S
16	m	51	Myocardial infarction Ventricular fibrillation	1	N	S
17	f	62	Coronary heart disease Ventricular fibrillation	9	N	NS
18	m	70	Coronary heart disease Ventricular fibrillation	1	N	S
19	f	62	Coronary heart disease Ventricular fibrillation	2	N	S
20	f	51	Myocardial infarction Ventricular fibrillation	7	Y	NS
21	f	69	Myocardial infarction Ventricular fibrillation	10	Y	S
22	m	72	Myocardial infarction Asystole	—	Y	NS
23	m	88	Myocardial infarction Ventricular fibrillation	4	Y	NS
24	f	82	Respiratory failure (COPD) Ventricular fibrillation	1	Y	NS
25	m	75	Coronary heart disease Ventricular fibrillation	1	Y	S

N: no; NS: nonsurvivor; S: survivor; U: unclear; Y: yes.

Table 2: Patient characteristics.

Patient characteristics	Number (no.)/value (mean ± SD; range)
Patient factors	
Age	63 ± 15 (35–90 years)
Gender (m/f)	19/6
Outcome (survivors/nonsurvivors)	15/10
Cause of cardiac arrest	
Cardiac disease	18
Respiratory failure	3
Lung embolism	1
Asphyxia	1
Trauma	1
Unknown	1
Initial cardiac rhythm	
Ventricular fibrillation	17
Asystole	8
Event factors	
Witnessed collapse	9
Bystander CPR	8
Time to ROSC in minutes	21 ± 13 (5–50 minutes)
Number of shocks ($n = 17$)	4 ± 3 (1–10)
Treatment	
Hypothermia/normothermia	6/7

were tested using ANOVA and post hoc nonparametric Mann–Whitney rank sum test. The results were declared as mean values with standard error of the mean (SEM) and were considered statistically significant for $p < 0.05$. Statistical analysis was performed using SigmaStat (version 4.0)/SigmaPlot (version 11.0).

3. Results

3.1. Clinical Baseline Characteristics. Twenty-five patients (63 ± 15 years; range: 35–90 years) who were resuscitated from OHCA were included (Table 1). MOF score was calculated in 15 patients detecting multiple organ failure in 60% (MOF < 4: $n = 6$; MOF ≥ 4: $n = 9$). 60% survived after cardiac arrest (survivors $n = 15$; nonsurvivors $n = 10$). TH was induced in six patients following a standardized protocol; seven patients were not admitted to any kind of TH treatment (normothermia: $n = 7$; hypothermia: $n = 6$). Detailed information regarding patient characteristics, cardiac arrest, and postresuscitation treatment are depicted in Table 2.

3.2. Time-Course of mRNA Expression Profiles. Gene expression dynamics of TNF-alpha, IL-8, IL-10, and IL-1ra compared to mRNA levels immediately after ROSC (A) are illustrated in Figure 1. Values are given in copies/μlRNA.

3.3. Proinflammatory Cytokines. Initially after cardiopulmonary resuscitation, TNF-alpha mRNA expression showed very high levels followed by continuous decrease during the course of time with a significant minimum after 24 h (889 ± 335 (A) versus 257 ± 74 (24 h); $p = 0.002$). IL-8 mRNA expression levels showed a peak immediately after cardiac arrest followed by a significant decrease with minimal levels after 12–72 h (1351 ± 489 (A) versus 71 ± 19 (12 h), 1351 ± 489 (A) versus 12 ± 2 (24 h), 1351 ± 489 (A) versus 13 ± 3 (48 h), and 1351 ± 489 (A) versus 12 ± 3 (72 h); $p < 0.001$).

3.4. Anti-Inflammatory Cytokines. IL-10 mRNA expression levels showed undulant low values with a gradual nonsignificant increase after 12 h followed by a nonsignificant decrease. IL-1ra mRNA expression levels started on a lower level immediately after CPR followed by a significant increase starting after 6 h (2629 ± 335 (A) versus 6940 ± 1055 (6 h), $p < 0.001$, 2629 ± 335 (A) versus 6144 ± 1055 (12 h), $p = 0.002$, and 2629 ± 335 (A) versus 5900 ± 948 (48 h); $p = 0.005$).

3.5. Gene Expression Levels and Clinical Outcome. IL-8, IL-10, and IL-1ra mRNA levels were higher in patients suffering from multiple organ failure without revealing statistically significant differences (Figure 2). Nonsurvivors expressed significantly higher IL-8 mRNA levels immediately after initiation of CPR when compared to survivors (719 ± 349 (survivors) versus 2300 ± 1157 (nonsurvivors); $p = 0.02$; Figure 3). IL-10 mRNA levels were significantly elevated after 48 h (23 ± 8 (survivors) versus 40 ± 13 (nonsurvivors); $p = 0.046$) and 72 h (16 ± 5 (survivors) versus 37 ± 9 (nonsurvivors); $p = 0.034$) in nonsurvivors. TNF-alpha and IL-1ra mRNA levels did not show any significant differences between the two groups.

3.6. Gene Expression Levels and Therapeutic Hypothermia. IL-1ra mRNA expression levels were higher in all patients following hypothermia, reaching significant higher levels after 48 h (3369 ± 640 (normothermia) versus 9629 ± 2330 (hypothermia); $p = 0.019$) and 72 h (3588 ± 1040 (normothermia) versus 10,886 ± 2183 (hypothermia); $p = 0.014$) in the hypothermia group (Figure 4). Patients treated with TH showed significantly higher IL-10 mRNA levels after 12 h when compared to normothermia group (20 ± 6 (normothermia) versus 48 ± 9 (hypothermia); $p = 0.028$). TNF-alpha and IL-8 mRNA levels did not show any significant changes.

4. Discussion

To our knowledge, this is the first study evaluating mRNA expression changes starting immediately after initiation of cardiopulmonary resuscitation. Via detecting very early mRNA expression changes of both pro- and anti-inflammatory cytokines, we could demonstrate a characteristic gene expression kinetic following cardiac arrest. Furthermore, we could detect significant cytokine alterations depending on clinical outcome directly after cardiac arrest. Therapeutic hypothermia leads to significant higher IL1-ra mRNA levels when compared to patients treated with normothermia.

4.1. Mediators of Interest. Several cytokines have already been identified to play a pivotal role in postresuscitation immunological changes and have therefore been chosen for the

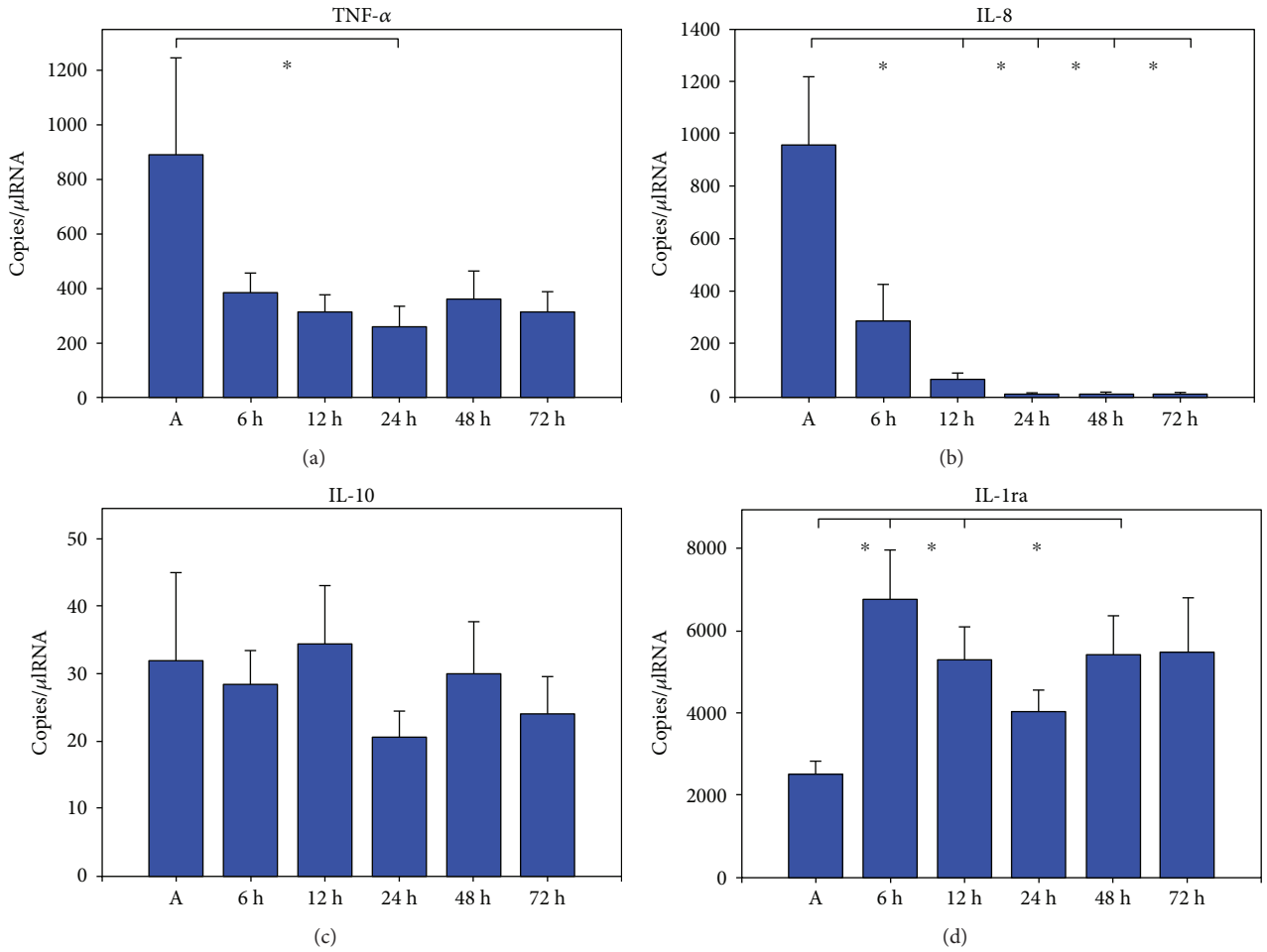

Figure 1: Plots of cytokine mRNA expression of TNF-alpha (a), IL-8 (b), IL-10 (c), and IL-1ra (d) 0 h–72 h after ROSC; bars represent mean values with error bars representing SEM. Kruskal-Wallis test and post hoc Dunn's test $^{*}p < 0.05$; all patients $n = 25$.

present investigation. TNF-alpha and interleukin-8 have a high amplifying potential regarding the primary systemic response after ROSC [2, 4, 20, 21]. Interleukin-10 is known as an essential PCAS-related anti-inflammatory cytokine controlling the downregulation of proinflammatory cytokines [13]. Anti-inflammatory IL-1ra has been focused in postresuscitation studies functioning as a marker of IL-1 activity [22, 23].

4.2. Results. The highlight of this investigation is the possibility to demonstrate such early mRNA expression changes in this vulnerable patient population following cardiopulmonary resuscitation. Thereby, we could show that TNF-alpha and IL-8 mRNA levels were expressively high immediately after cardiac arrest followed by a significant decrease over the next 72 hours. These results confirm previous findings demonstrating a rapid upregulation of proinflammatory markers after ROSC. In this context, Adrie et al. and Ito et al. reported similar time-course changes of TNF-alpha and IL-8 in their cohorts showing a very early cytokine release after the ischemic event [20, 24]. However, in contrast to our study, both groups only started their observations when patients were admitted to the hospital/intensive care unit. In their experimental setting, Niemann et al. could detect early changes showing increased TNF-alpha levels within 30 minutes after ROSC [25]. Regarding the characteristic cytokine release, our observations are in line with Adrie et al. who also documented a subsequent decrease of TNF-alpha and IL-8 in the later course of the following three days [24]. Bro-Jeppesen et al. also found high initial TNF-alpha concentrations followed by a significant decrease within the first 72 hours after ROSC [3]. On the anti-inflammatory side, IL-10 expression was constantly low in our cohort whereas IL-1ra levels started on a lower baseline level and increased significantly beginning 6 h after ROSC. In summary, cytokine mRNA expression underlie an immediate characteristic time-course in our cohort showing a significant downregulation of pro- and upregulation of anti-inflammatory cytokines reflecting the coexistence of both in case of PCAS. This might be understood as an attempt of the postresuscitation organism to attenuate the progressive inflammation mediated by proinflammatory cytokines by raising the anti-inflammatory immune defense [26]. In contrast to numerous studies that investigate cytokine changes in serum or plasma, a major strength of this work is the evaluation of mRNA expression changes. This is based on the idea that changes

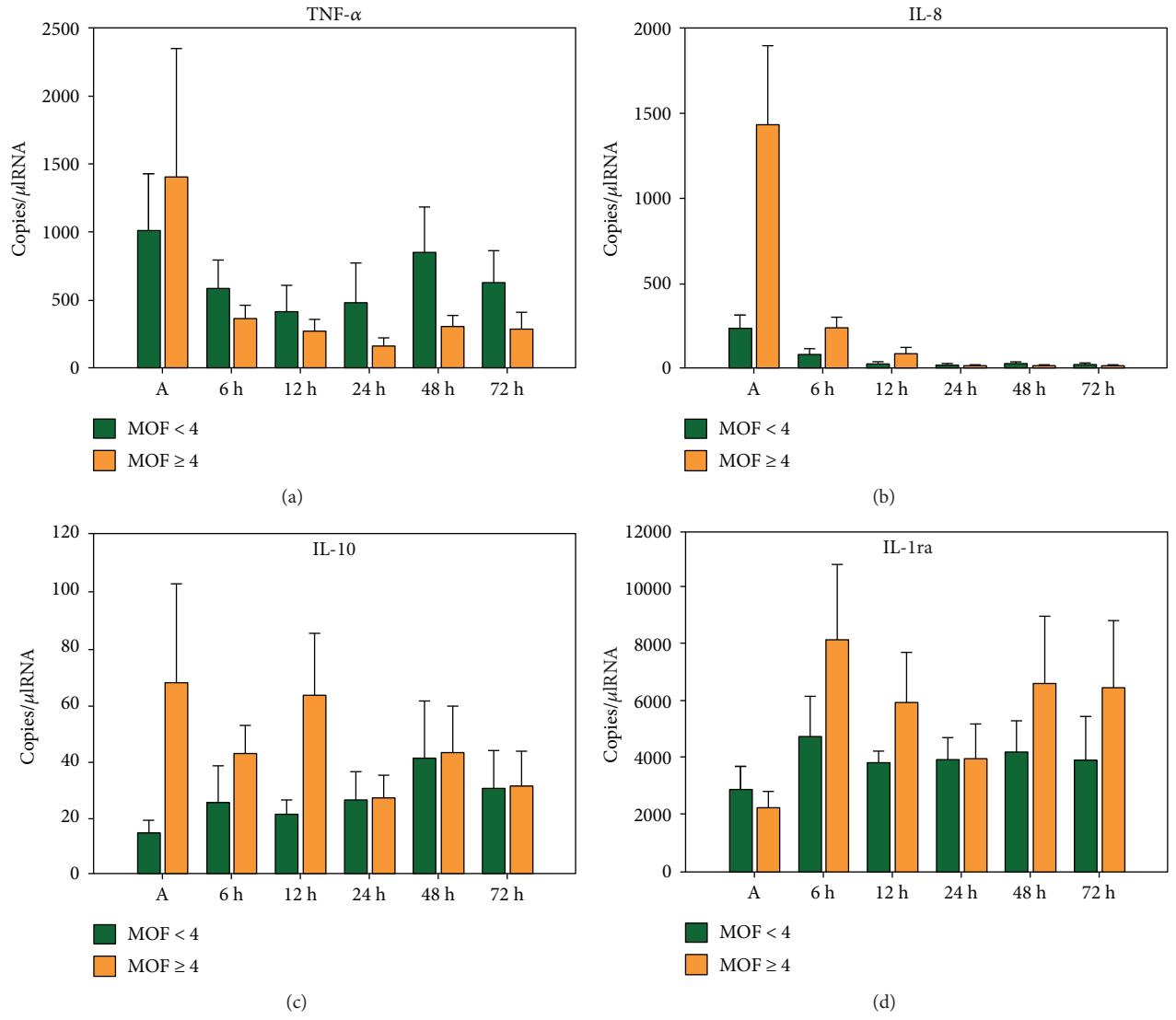

Figure 2: Plots of comparison between mRNA levels of TNF-alpha (a), IL-8 (b), IL-10 (c), and IL-1ra (d) and multiple organ failure assessed by multiple organ failure score (MOF score) 0 h–72 h after cardiac arrest; bars represent mean values with error bars representing SEM. ANOVA and post hoc Mann–Whitney rank sum test; MOF < 4 $n = 6$; MOF ≥ 4 $n = 9$.

on genome level can improve the understanding of underlying immune functionality. To our knowledge, there are solely some experimental studies available that evaluate mRNA expression levels after cardiac arrest. Meybohm et al. demonstrated a similar upregulation of TNF-alpha, ICAM-1, and IL-1b mRNA levels in cerebral tissue [14]. In contrast, Sipos et al. interpreted the downregulation of TNF-alpha and IL-2 mRNA levels in their study as an immune paralyze after cardiac arrest [1]. Major strength of these two studies is the evaluation of both mRNA and protein levels. The missing protein analysis might thereby be seen as a limitation of our work as mRNA levels do not necessarily correlate with protein levels. Particularly, IL-10 is known to underlie posttranscriptional regulation processes articulately influencing later protein levels. This might explain the diverse data concerning IL-10 expression levels in our series. However, one of the main advantages in studying gene expression profiles is the detection of very early transcriptome changes. By evaluating these changes starting in a preclinical setting, we could demonstrate that these fundamental functional immunological changes already start immediately after cardiac arrest.

4.3. Outcome. Several groups elucidated the correlation of PCAS-related cytokine changes and clinical outcome. Our results are in line with previous findings as we could detect a significant upregulation of IL-8 mRNA levels in nonsurvivors versus survivors immediately after cardiac arrest [20]. This phenomenon is only detectable in the very early postresuscitation period followed by a downregulation of IL-8 cytokine expression in both survivors and nonsurvivors. Similarly, TNF-alpha showed higher initial mRNA levels in nonsurvivors but did not reveal statistical significance. Whereas the proinflammatory immune reaction started immediately after cardiac arrest in our study, anti-inflammatory cytokine dynamics started minimally temporally delayed demonstrating significant higher IL-10

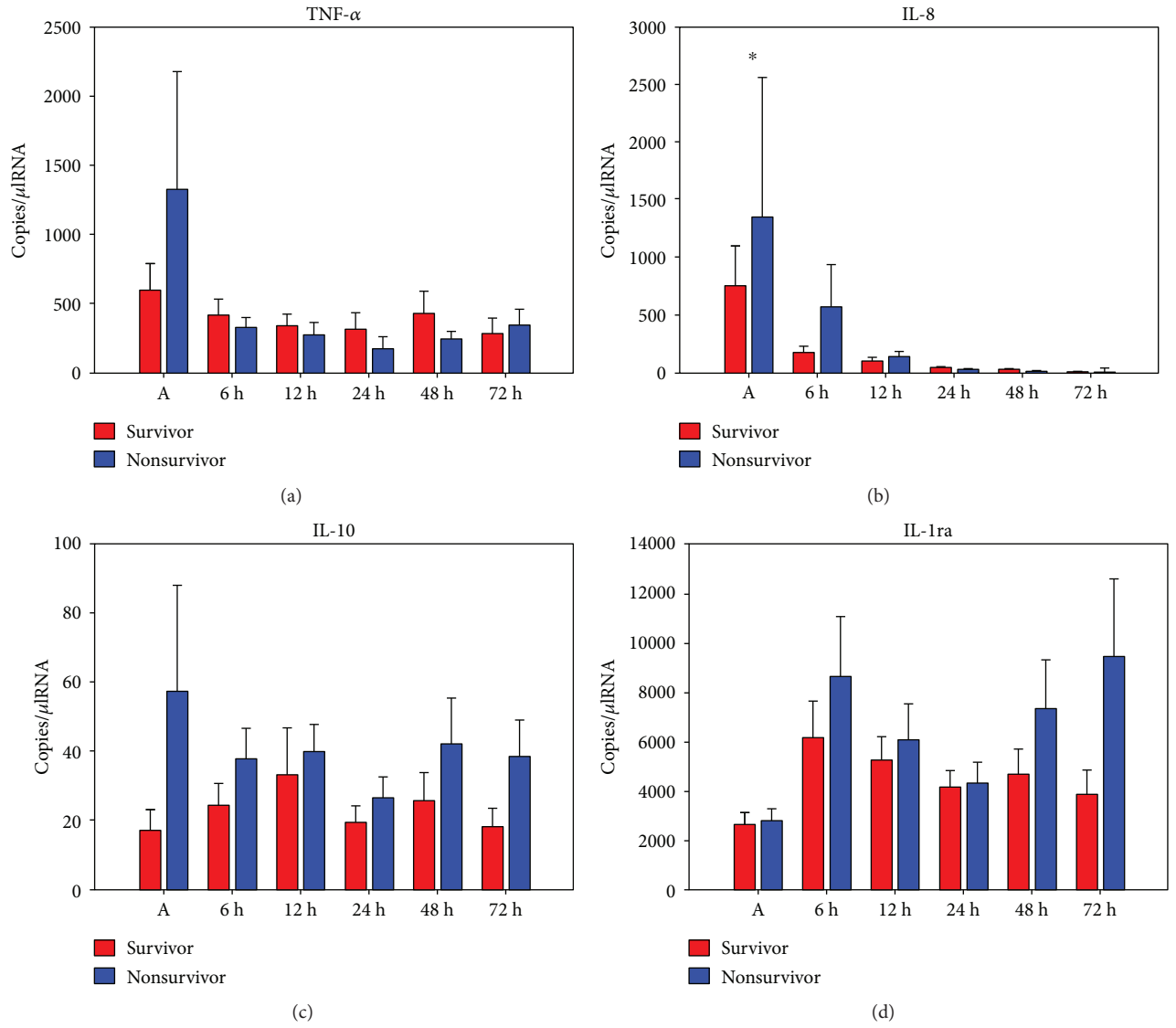

Figure 3: Plots of comparison between mRNA levels of TNF-alpha (a), IL-8 (b), IL-10 (c), and IL-1ra (d) and 30-day survival 0 h–72 h after cardiac arrest; bars represent mean values with error bars representing SEM. ANOVA and post hoc Mann–Whitney rank sum test $^{*}p < 0.05$; survivors ($n = 15$) and nonsurvivors ($n = 10$).

mRNA levels 48 h and 72 h after the beginning of CPR in nonsurvivors. However, IL-10 mRNA levels were very low in our cohort during the whole observation period. Therefore, the value of these results remains questionable. Peberdy et al. recently documented significant higher IL-1ra, IL-6, IL-8, and IL-10 levels in nonsurvivors and patients with poor functional outcome which is similar to our results showing higher IL-1ra expression levels in nonsurvivors [21]. However, IL-1ra levels did not reveal statistical significance presumably on account of the small sample size. With regard to the devolvement of multiple organ failure, no statistical significance could be detected between the two groups. By trend, patients suffering from multiple organ failure showed higher mRNA levels of IL-8, IL-10, and IL-1ra.

4.4. Impact of Therapeutic Hypothermia. By studying early cytokine changes after cardiac arrest, we could further show a significant increase of anti-inflammatory IL-1ra mRNA levels in patients treated with TH. Simultaneously, proinflammatory TNF-alpha mRNA levels were lower in the hypothermia group by trend without revealing statistical significance. From our point, these findings might support the relationship between postresuscitation inflammatory parameters and induced hypothermia. This temperature-related effect might be interpreted as a beneficial impact of TH on cytokine dynamics that attenuates the proinflammatory "injury" by raising anti-inflammatory mechanisms. Major strength of this study is the normothermic control group as the study was conducted when TH was not yet implemented in the guidelines. Based on several studies, hypothermia is strongly advocated in postresuscitation patients to reduce PCAS-related neurologic dysfunction and improve outcome [5–7]. Nevertheless, the impact on the enhanced systemic immune reaction has not been fully understood, as available

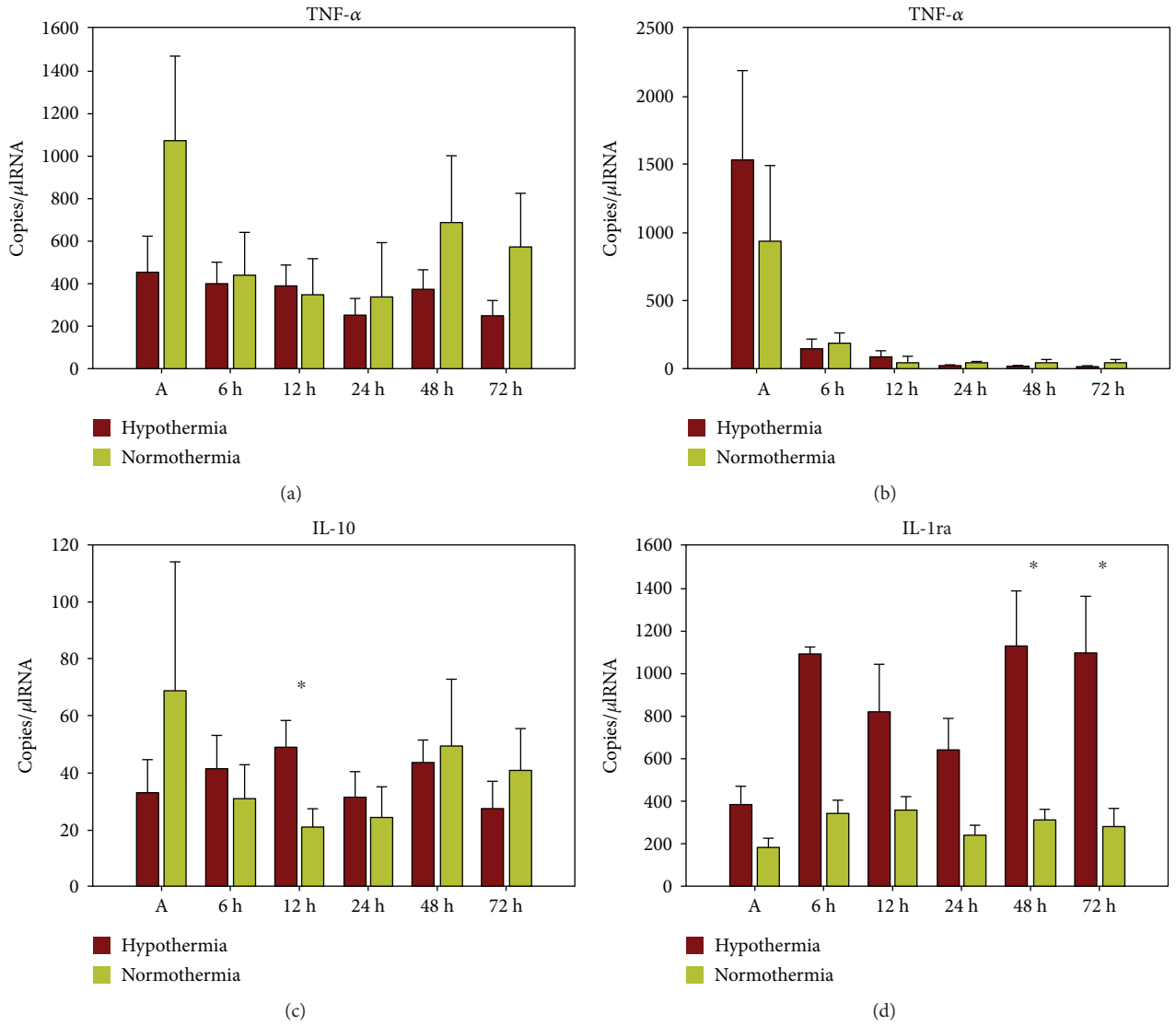

FIGURE 4: Plots of comparison between mRNA levels of TNF-alpha (a), IL-8 (b), IL-10 (c), and IL-1ra (d) and therapeutic hypothermia 0 h–72 h after cardiac arrest; bars represent mean values with error bars representing SEM. ANOVA and post hoc Mann–Whitney rank sum test $^{*}p < 0.05$; hypothermia ($n = 6$); normothermia ($n = 7$).

data is still conflicting. Meybohm et al. demonstrated a significant reduction of elevated proinflammatory cytokines due to hypothermia [14]. They concluded a positive influence on outcome via reduced apoptosis rates and proinflammatory cytokine expression. Similar inhibitory effects of TH represented by a decrease of inflammatory response have been stated by Yanagawa et al. [27]. In their study, the ischemic brain incubated at 33°C showed decreased IL-6 levels when compared to 36°C. Contrary, Sipos et al. could not demonstrate any effect of TH on cytokine expressions [1]. Regarding the available clinical studies, results and conclusions are widely diverging. Fries et al. demonstrated a suppressed release of CRP but not IL-6 by induction of TH [13]. Aibiki et al. have demonstrated a decreased proinflammatory immune reaction and improved outcome as a result of TH [28]. In contrast, Bisschops et al. concluded that hypothermia could not prevent high proinflammatory cytokine levels [29]. Similarly, Callaway et al. did not find any association between cytokine changes and neurologic recovery after hypothermia and concluded that damping the inflammatory response might not be the reason for the beneficial effect of TH [30]. Bro-Jeppesen et al. demonstrated, in their randomized controlled trial, that mediators of the pro- and anti-inflammatory response were not significantly influenced by TTM at 33°C compared to 36°C [15]. When evaluating this conflicting data, the majority of publications suggest that the benefit of hypothermia is the "pan inhibition" of ischaemia-induced damaging pathways. However, Zhao et al. recently emphasized another potential effectiveness of TH. They hypothesized that the beneficial impact might result from the induction of protective genes and not only from a down streaming of detrimental ones [31]. Referring to this, Hicks and colleagues found that experimentally induced hypothermia was associated with distinct changes

of stress-induced protein expressions [8]. Regarding our results, we therefore conclude that TH reduces proinflammatory mRNA cytokine expression as seen by lower TNF-alpha levels and a significant upregulation of IL-1ra expression, which becomes especially remarkable after 6–72 h, potentially representing a protective anti-inflammatory immune response. Nevertheless, the positive effect of TH via modification of anti-inflammatory cytokine expression profiles remains unclear and might therefore only serve as an attempt to explain our results. As IL-1ra mRNA levels were—by tendency—also higher in nonsurvivors and patients suffering from MOF, TH might not be the only reason for the anti-inflammatory response. Due to the small sample size, further subgroup analysis (survivors versus nonsurvivors with or without TH) was not possible.

4.5. Limitations of This Study. Main limitations of our work are the small sample size and heterogeneous cohort due to the challenging clinical setting. Our emergency physician collected the first blood sample preclinical immediately after initiation of CPR in order to evaluate cytokine changes as early as possible. Afterwards, patients were admitted to various hospitals in town leading to nonstandardized treatment and difficulties in completing all data sets. The induction of TH varied significantly in the different hospitals. In order to reduce heterogeneity in this subcohort, only patients who were treated according to the same protocol were included in TH group leading to small subgroups.

5. Conclusion

The very early postcardiac arrest interval is characterized by a significant pro- and anti-inflammatory cytokine expression time-course. Nonsurvivors express significantly higher IL-8 mRNA levels immediately after cardiac arrest. Following successful resuscitation, therapeutic hypothermia is accompanied by a significant upregulation of IL-1ra mRNA potentially suggesting a protective effect of TH by raising the anti-inflammatory postresuscitation immune defense.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

[1] W. Sipos, C. Duvigneau, F. Sterz et al., "Changes in interleukin-10 mRNA expression are predictive for 9-day survival of pigs in an emergency preservation and resuscitation model," *Resuscitation*, vol. 81, no. 5, pp. 603–608, 2010.

[2] C. Adrie, I. Laurent, M. Monchi, A. Cariou, J.-F. Dhainaou, and C. Spaulding, "Postresuscitation disease after cardiac arrest: a sepsis-like syndrome?," *Current Opinion in Critical Care*, vol. 10, no. 3, pp. 208–212, 2004.

[3] J. Bro-Jeppesen, J. Kjaergaard, P. Stammet et al., "Predictive value of interleukin-6 in post-cardiac arrest patients treated with targeted temperature management at 33°C or 36°C," *Resuscitation*, vol. 98, pp. 1–8, 2016.

[4] J. P. Nolan, R. W. Neumar, C. Adrie et al., "Post-cardiac arrest syndrome: epidemiology, pathophysiology, treatment, and prognostication: a scientific statement from the International Liaison Committee on Resuscitation; the American Heart Association Emergency Cardiovascular Care Committee; the Council on Cardiovascular Surgery and Anesthesia; the Council on Cardiopulmonary, Perioperative, and Critical Care; the Council on Clinical Cardiology; the Council on Stroke (part II)," *International Emergency Nursing*, vol. 18, pp. 8–28, 2010.

[5] S. A. Bernard, T. W. Gray, M. D. Buist et al., "Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia," *The New England Journal of Medicine*, vol. 346, no. 8, pp. 557–563, 2002.

[6] C. D. Deakin, J. P. Nolan, J. Soar et al., "European Resuscitation Council Guidelines for Resuscitation 2010 Section 4. Adult advanced life support," *Resuscitation*, vol. 81, no. 10, pp. 1305–1352, 2010.

[7] Hypothermia after Cardiac Arrest Study Group, "Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest," *New England Journal of Medicine*, vol. 346, pp. 549–556, 2002.

[8] S. D. Hicks, D. B. DeFranco, and C. W. Callaway, "Hypothermia during reperfusion after asphyxial cardiac arrest improves functional recovery and selectively alters stress-induced protein expression," *Journal of Cerebral Blood Flow & Metabolism*, vol. 20, no. 3, pp. 520–530, 2000.

[9] F. Colbourne, G. Sutherland, and D. Corbett, "Postischemic hypothermia. A critical appraisal with implications for clinical treatment," *Molecular Neurobiology*, vol. 14, no. 3, pp. 171–201, 1997.

[10] M. D. Ginsberg, L. L. Sternau, M. Y. Globus, W. D. Dietrich, and R. Busto, "Therapeutic modulation of brain temperature: relevance to ischemic brain injury," *Cerebrovascular and Brain Metabolism Reviews*, vol. 4, no. 3, pp. 189–225, 1992.

[11] R. E. Hoesch and R. G. Geocadin, "Therapeutic hypothermia for global and focal ischemic brain injury—a cool way to improve neurologic outcomes," *The Neurologist*, vol. 13, no. 6, pp. 331–342, 2007.

[12] K. H. Polderman, "Mechanisms of action, physiological effects, and complications of hypothermia," *Critical Care Medicine*, vol. 37, no. 7, pp. S186–S202, 2009.

[13] M. Fries, C. Stoppe, D. Brücken, R. Rossaint, and R. Kuhlen, "Influence of mild therapeutic hypothermia on the inflammatory response after successful resuscitation from cardiac arrest," *Journal of Critical Care*, vol. 24, no. 3, pp. 453–457, 2009.

[14] P. Meybohm, M. Gruenewald, K. D. Zacharowski et al., "Mild hypothermia alone or in combination with anesthetic post-conditioning reduces expression of inflammatory cytokines in the cerebral cortex of pigs after cardiopulmonary resuscitation," *Critical Care*, vol. 14, no. 1, article R21, 2010.

[15] J. Bro-Jeppesen, J. Kjaergaard, M. Wanscher et al., "The inflammatory response after out-of-hospital cardiac arrest is not modified by targeted temperature management at 33°C or 36°C," *Resuscitation*, vol. 85, no. 11, pp. 1480–1487, 2014.

[16] M. A. Peberdy, M. Cretikos, B. S. Abella et al., "Recommended guidelines for monitoring, reporting, and conducting research on medical emergency team, outreach, and rapid response systems: an Utstein-style scientific statement: a scientific statement from the International Liaison Committee on Resuscitation (American Heart Association, Australian Resuscitation Council, European Resuscitation Council, Heart and Stroke Foundation of Canada, InterAmerican Heart

Foundation, Resuscitation Council of Southern Africa, and the New Zealand Resuscitation Council); the American Heart Association Emergency Cardiovascular Care Committee; the Council on Cardiopulmonary, Perioperative, and Critical Care; and the Interdisciplinary Working Group on Quality of Care and Outcomes Research," *Circulation*, vol. 116, no. 21, pp. 2481–2500, 2007.

[17] G. D. Perkins, I. G. Jacobs, V. M. Nadkarni et al., "Cardiac arrest and cardiopulmonary resuscitation outcome reports: update of the Utstein Resuscitation Registry Templates for Out-of-Hospital Cardiac Arrest: a statement for healthcare professionals from a task force of the International Liaison Committee on Resuscitation (American Heart Association, European Resuscitation Council, Australian and New Zealand Council on Resuscitation, Heart and Stroke Foundation of Canada, InterAmerican Heart Foundation, Resuscitation Council of Southern Africa, Resuscitation Council of Asia); and the American Heart Association Emergency Cardiovascular Care Committee and the Council on Cardiopulmonary, Critical Care, Perioperative and Resuscitation," *Circulation*, vol. 132, no. 13, pp. 1286–1300, 2015.

[18] R. J. Goris, T. P. te Boekhorst, J. K. Nuytinck, and J. S. Gimbrère, "Multiple-organ failure. Generalized autodestructive inflammation?," *Archives of Surgery*, vol. 120, no. 10, pp. 1109–1115, 1985.

[19] R. Lefering, J. Goris, E. van Nieuwenhoven, and E. Neugebauer, "Revision of the multiple organ failure score," *Langenbeck's Archives of Surgery*, vol. 387, no. 1, pp. 14–20, 2002.

[20] T. Ito, D. Saitoh, K. Fukuzuka et al., "Significance of elevated serum interleukin-8 in patients resuscitated after cardiopulmonary arrest," *Resuscitation*, vol. 51, no. 1, pp. 47–53, 2001.

[21] M. A. Peberdy, L. W. Andersen, A. Abbate et al., "Inflammatory markers following resuscitation from out-of-hospital cardiac arrest—a prospective multicenter observational study," *Resuscitation*, vol. 103, pp. 117–124, 2016.

[22] B. W. Van Tassell, S. Toldo, E. Mezzaroma, and A. Abbate, "Targeting interleukin-1 in heart disease," *Circulation*, vol. 128, no. 17, pp. 1910–1923, 2013.

[23] C. A. Dinarello, "Historical insights into cytokines," *European Journal of Immunology*, vol. 37, Supplement S1, pp. S34–S45, 2007.

[24] C. Adrie, M. Adib-Conquy, I. Laurent et al., "Successful cardiopulmonary resuscitation after cardiac arrest as a "sepsis-like" syndrome," *Circulation*, vol. 106, no. 5, pp. 562–568, 2002.

[25] J. T. Niemann, S. T. Youngquist, A. P. Shah, J. L. Thomas, and J. P. Rosborough, "TNF-α blockade improves early post-resuscitation survival and hemodynamics in a swine model of ischemic ventricular fibrillation," *Resuscitation*, vol. 84, no. 1, pp. 103–107, 2013.

[26] C. Adrie and M. R. Pinsky, "The inflammatory balance in human sepsis," *Intensive Care Medicine*, vol. 26, no. 4, pp. 364–375, 2000.

[27] Y. Yanagawa, M. Kawakami, and Y. Okada, "Moderate hypothermia alters interleukin-6 and interleukin-1α reactions in ischemic brain in mice," *Resuscitation*, vol. 53, no. 1, pp. 93–99, 2002.

[28] M. Aibiki, S. Maekawa, S. Ogura, Y. Kinoshita, N. Kawai, and S. Yokono, "Effect of moderate hypothermia on systemic and internal jugular plasma IL-6 levels after traumatic brain injury in humans," *Journal of Neurotrauma*, vol. 16, no. 3, pp. 225–232, 1999.

[29] L. L. A. Bisschops, J. G. van der Hoeven, T. E. Mollnes, and C. W. E. Hoedemaekers, "Seventy-two hours of mild hypothermia after cardiac arrest is associated with a lowered inflammatory response during rewarming in a prospective observational study," *Critical Care*, vol. 18, no. 5, p. 546, 2014.

[30] C. W. Callaway, J. C. Rittenberger, E. S. Logue, and M. J. McMichael, "Hypothermia after cardiac arrest does not alter serum inflammatory markers," *Critical Care Medicine*, vol. 36, no. 9, pp. 2607–2612, 2008.

[31] H. Zhao, C. S. Li, P. Gong et al., "Molecular mechanisms of therapeutic hypothermia on neurological function in a swine model of cardiopulmonary resuscitation," *Resuscitation*, vol. 83, no. 7, pp. 913–920, 2012.