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?

PROGNOSTIČKA VREDNOST I DNEVNI TREND INTERLEUKINA-6, EKSPRESIJE CD64 NA NEUTROFILIMA, C-REAKTIVNOG PROTEINA I LIPOPOLISAHARID-VEZUJUĆEG PROTEINA KOD KRITIČNO OBOLELIH PACIJENATA: POUZDANI PREDIKTORI ISHODA ILI NE?

Dragan Djordjevic1,2, Janko Pejovic3,2, Maja Surbatovic1,2, Jasna Jevdijc4,5, Sonja Radakovic6,2, Milic Veljovic1,2, Aneta Peric7,2, Tamara Andjelic3, Nada Popovic8,9

1Clinic of Anesthesiology and Intensive Therapy, Military Medical Academy, Belgrade, Serbia
2Faculty of Medicine of the Military Medical Academy, University of Defence, Belgrade, Serbia
3Institute of Medical Biochemistry, Military Medical Academy, Belgrade, Serbia
4Faculty of Medical Sciences, University of Kragujevac, Serbia
5Clinical Center Kragujevac, Serbia
6Sector of Preventive Medicine, Military Medical Academy, Belgrade, Serbia
7Sector for pharmacy, Military Medical Academy, Belgrade, Serbia
8School of Medicine, University of Belgrade, Serbia
9Clinic for Burns, Plastic and Reconstructive Surgery, Belgrade, Serbia

Summary

Background: Severe sepsis and/or trauma complicated by multiple organ dysfunction syndrome are the leading causes of death in critically ill patients. The aim of this prospective single-centre study was to assess the prognostic value and daily trend of interleukin-6 (IL-6), neutrophil CD64 expression, C-reactive protein (CRP) and lipopolysaccharide-binding protein (LBP) regarding outcome in critically ill patients with severe trauma and/or severe sepsis. Outcome measure was hospital mortality.

Methods: One hundred and two critically ill patients admitted to the intensive care unit of a tertiary university hospital were enrolled in this prospective study. Blood samples were collected on admission (day 1), days 2 and 3.

Results: CD64 index was 1.6-fold higher on day 1 and 1.78-fold higher on day 2 in non-survivors (p<0.05). The area under the curve (AUC) for the CD64 index on day 1 for outcome was 0.727. At a cut-off level of 2.80 sensitivity was 75% and specificity was 65%. Patients with CD64 index level over 2.80 had 2.4 times higher risk of hospital mortality.

Conclusion: CD64 index was a good predictor of outcome. AUC/ROC values for IL-6, CRP and LBP were <0.55, indicating that these biomarkers were not good predictors of outcome.

Address for correspondence:
Professor Maja Surbatovic MD, PhD
e-mail: maja.surbatovic@gmail.com
Phone: 064 47 27 306
on day 1 higher than 2.80 had 2.4-fold higher probability of dying. Odds ratio was 2.40; 95% CI 0.60–9.67.

**Conclusions:** CD64 index on day 1 is a fairly good predictor of outcome. AUCs for IL-6, CRP and LBP were < 0.55, suggesting these biomarkers failed to predict outcome.

**Keywords:** biomarkers, critical care, leukocytes, outcome, prognosis

**Introduction**

Severe sepsis and/or trauma complicated by multiple organ dysfunction syndrome (MODS) are the leading causes of death in critically ill patients. Major determinant of outcome is intensity of insult as well as immuno-inflammatory response (1). Genetic predisposition (2) and high level of variability in circulating levels of various mediators (3–5) contribute to the complexity of the inflammatory response.

Interleukin (IL-6) is a proinflammatory cytokine with a pivotal role in acute-phase protein synthesis induction. Consequently, peak plasma levels of IL-6 are observed earlier than the highest levels of acute-phase proteins. Two of numerous acute-phase proteins, lipopolysaccharide-binding protein (LBP) and the well-known C-reactive protein (CRP), are interesting in terms of being markers of infection. LBP is involved in innate immune response with the specific role of binding to lipopolysaccharide. This complex then interacts with CD14 receptor and Toll-like receptor (TLR)-4 on monocytes/macrophages resulting in cytokine cascade initiation. LBP has two interesting features. First, although it binds to lipopolysaccharide from Gram-negative bacteria, this acute-phase protein is also elevated in Gram-positive sepsis. Second, the half-life of LBP is longer in comparison with a pivotal role in acute-phase protein synthesis induction. Consequently, peak plasma levels of IL-6 are observed earlier than the highest levels of acute-phase proteins. Two of numerous acute-phase proteins, lipopolysaccharide-binding protein (LBP) and the well-known C-reactive protein (CRP), are interesting in terms of being markers of infection. LBP is involved in innate immune response with the specific role of binding to lipopolysaccharide. This complex then interacts with CD14 receptor and Toll-like receptor (TLR)-4 on monocytes/macrophages resulting in cytokine cascade initiation. LBP has two interesting features. First, although it binds to lipopolysaccharide from Gram-negative bacteria, this acute-phase protein is also elevated in Gram-positive sepsis. Second, the half-life of LBP is longer in comparison with cytokines induced by it (6). CRP plays an important role in immune response to insult, notably enhancing phagocytosis by binding to polysaccharides of microbes, and evolutionary is conserved and stable. Its peak concentration in plasma is usually 12 hours after LBP. Liver is mainly responsible for CRP synthesis (7). Quantitative neutrophil CD64 (high affinity Fcγ receptor) expression starts from less than 2000 sites per cell and becomes up-regulated in patients with systemic inflammatory response syndrome (8, 9). Although these biomarkers are not novel, in literature we found conflicting findings for each of them regarding outcome in critically ill patients.

The aim of this prospective single-centre study was to assess the prognostic value and daily trend of IL-6, neutrophil CD64, CRP and LBP regarding outcome in critically ill patients with severe trauma and/or severe sepsis. Outcome measure was hospital mortality.

**Materials and Methods**

**Patients**

This prospective study was conducted in a tertiary university hospital (Military Medical Academy, Belgrade, Serbia). Approval in concordance with the Declaration of Helsinki was obtained from the local ethics committee and informed consent from the patient or a first-degree relative. One hundred and two critically ill patients, admitted to the surgical intensive care unit (SICU) from August 2010 until March 2012 were enrolled. Blood samples were collected on admission (day 1), day 2 and day 3 from all patients with either severe sepsis or severe trauma with and without secondary sepsis. Sequential Organ Failure Assessment (SOFA) score (10), Simplified Acute Physiology Score (SAPS) II (11) and Acute Physiology and Chronic Health Evaluation (APACHE) II score (12) were calculated and recorded within the first 24 hours after admission to the SICU.

Sepsis patients were enrolled if they had fulfilled the current diagnostic criteria for severe sepsis (sepsis-induced tissue hypoperfusion or organ dysfunction) (13). For patients with blunt and/or penetrating trauma, Injury Severity Score – ISS (determined using Abbreviated Injury Scale – AIS) was calculated and recorded.

**Sampling and analysis**

Patient’s venous blood was drawn by trained, qualified phlebotomists. Two blood samples were taken from each patient: one serum tube (with clot activator) and one EDTA sample tube. Serum specimens were allowed to clot for 30 minutes in a vertical position and then centrifuged at 1300 RCF for 10 minutes. Quantitative measurements of CRP, IL-6 and LBP were performed from serum specimens, while neutrophil CD64 assay was measured from EDTA anticoagulated whole blood. All laboratory tests were performed within 3 hours of the patient draw time.

**IL-6 and LBP measurements**

Quantitative measurements of IL-6 and LBP were performed on a Siemens Immulite 2000 by solid-phase, enzyme-labeled, chemiluminescent immunometric assays. The instrument uses beads coated with appropriate monoclonal murine antibodies. The sample was incubated with an alkaline phosphatase-labeled reagent. Four washing cycles allowed no residual unbound label. Light was emitted when the chemiluminescent substrate reacted with alkaline phosphatase. The amount of emitted light was proportional to the IL-6 (LBP) concentration (14, 15).
Neutrophil CD64 assay

Quantitative measurement of neutrophil CD64 expression was done by an Abbott Cell-Dyne Sapphire hematology analyzer with the Trillium Diagnostic Leuko 64 assay kit. The principle of the assay is flow cytometry. The assay contains a mixture of monoclonal antibodies to two different epitopes of CD64 (clones 22 and 32.2) and monoclonal antibody to CD163 (clone Mac2-158). Fifty µL of reagent A and 50 µL EDTA whole blood were mixed, incubated for 10 min. at room temperature and loaded into the analyzer. The results were calculated and reported as CD64 index by the Leuko64 QuantiCALC software, which used cluster-finding algorithms to locate lymphocytes, monocytes and neutrophils (16).

CRP measurement

Quantitative measurements of C-Reactive Protein were performed using the Siemens Dimension RxL Max analyzer, by particle enhanced turbidimetric immunoassay (PETIA). Anti-CRP coated particles of the reagent in the presence of CRP in the sample aggregate, causing an increase in turbidity. The increase in turbidity (at 340 nm) is proportional to CRP concentration in the sample.

Statistical analysis

All results are reported as mean ± SD. Statistical analysis of the results was performed with Mann-Whitney U – Wilcoxon Rank Sum W test, Pearson’s Chi-Square test and Student’s t-test. Receiver operator curves were generated to determine cut-off values for optimal sensitivity and specificity of the IL-6, CRP, LBP and CD64 levels for outcome prediction. The prognostic accuracy of biomarkers was expressed as the area under the ROC curve (AUC) and the 95% confidence interval (CI). The AUC defines the probability for correct discrimination between survivors and non-survivors. Statistical Package SPSS for Windows (version 7.2; Chicago, Illinois, USA) was used. Differences between groups were considered to be significant at p<0.05 and highly significant at p<0.01.

Results

Demographic data of the patients are shown in Table I. Profiles of IL-6, CD64, CRP and LBP from day 1 to day 3 are shown in Table II.

<table>
<thead>
<tr>
<th>Table I Demographic data.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of patients</td>
</tr>
<tr>
<td>Age (median, range)</td>
</tr>
<tr>
<td>Sex, n (%)</td>
</tr>
<tr>
<td>male</td>
</tr>
<tr>
<td>female</td>
</tr>
<tr>
<td>Simplified Acute Physiology Score II – SAPS II score, mean ± SD</td>
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<tr>
<td>Acute Physiology and Chronic Health Evaluation II – APACHE II score, mean ± SD</td>
</tr>
<tr>
<td>Sequential Organ Failure Assessment – SOFA score, mean ± SD</td>
</tr>
<tr>
<td>Reason for ICU admission, n (%)</td>
</tr>
<tr>
<td>Severe trauma (ISS 28.73 ± 9.40)</td>
</tr>
<tr>
<td>Severe trauma and secondary sepsis</td>
</tr>
<tr>
<td>Severe sepsis due to peritonitis</td>
</tr>
<tr>
<td>pancreatitis</td>
</tr>
<tr>
<td>other causes</td>
</tr>
<tr>
<td>Blood cultures, n (%)</td>
</tr>
<tr>
<td>Gram-positive</td>
</tr>
<tr>
<td>Gram-negative</td>
</tr>
<tr>
<td>Mixed</td>
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<tr>
<td>Fungi</td>
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<tr>
<td>Sterile</td>
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<tr>
<td>Mortality, n (%)</td>
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</tbody>
</table>

| Table II Profiles of IL-6, CD64, CRP and LBP from day 1 to day 3. |
|--------------------------|--------------------------|--------------------------|--------------------------|
| Parameter (Mean±SD)      | Day 1                    | Day 2                    | Day 3                    | p          |
| IL-6 (pg/mL)             | 595.74±278.08            | 212.30±128.17            | 151.98±115.84            | Z=–2.595; p<0.01 (day 1 vs day 2) |
| CD64 index               | 4.54±3.25                | 4.47±2.77                | 3.99±2.25                | Z=–1.965; p<0.05 (day 2 vs day 3) |
| CRP (mg/L)               | 120.82±55.83             | 143.43±71.55             | 125.04±57.76             | n.s.      |
| LBP (µg/mL)              | 36.80±17.34              | 34.90±15.37              | 37.33±17.03              | n.s.      |
Table III Profiles of IL-6, CD64, CRP and LBP from day 1 to day 3 according to outcome.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 1 survivors</th>
<th>Day 1 non-survivors</th>
<th>Day 2 survivors</th>
<th>Day 2 non-survivors</th>
<th>Day 3 survivors</th>
<th>Day 3 non-survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/mL)</td>
<td>400.55±216.39</td>
<td>764.00±478.80</td>
<td>153.68±98.15</td>
<td>268.47±101.78</td>
<td>96.27±64.68</td>
<td>197.85±106.47</td>
</tr>
<tr>
<td>CD64 index</td>
<td>3.49±2.23</td>
<td>5.64±3.82*</td>
<td>3.10±1.01</td>
<td>5.54±3.25*</td>
<td>3.01±1.23</td>
<td>4.75±2.63</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>157.96±83.32</td>
<td>143.37±86.69</td>
<td>141.91±69.84</td>
<td>160.47±88.46</td>
<td>151.42±46.39</td>
<td>131.40±72.42</td>
</tr>
<tr>
<td>LBP (µg/mL)</td>
<td>27.25±11.10</td>
<td>35.99±18.54</td>
<td>30.48±10.75</td>
<td>35.10±17.37</td>
<td>32.71±11.54</td>
<td>36.36±25.01</td>
</tr>
</tbody>
</table>

*survivors vs. non-survivors, p<0.05

Figure 1 Receiver operator curve for the CD64 index levels on day 1.

Figure 2 Values of IL-6 from day 1 to day 3 according to outcome.

Figure 3 Values of CD64 index from day 1 to day 3 according to outcome.

Figure 4 Values of CRP from day 1 to day 3 according to outcome.
because it is still rather new and not routinely determined. In our study, IL-6 was not a reliable predictor of outcome, but the daily trend of this potent proinflammatory mediator was interesting: overall, there was a trend of decrease with a statistically highly significant difference between day 1 and day 2. Mean value of IL-6 was highest on day 1, 595.74 pg/mL; on day 2 it was 212.3 pg/mL, and it was lowest on day 3, 151.98 pg/mL. The decrease in IL-6 circulating values on day 2 was much more pronounced in the non-survivors. A similar trend of IL-6 decrease from admission to day 3 and day 7 was found in the study of Miguel-Bayarri et al. (17) in a cohort of 81 septic patients. In their multivariate analysis with mortality as the dependent variable, they found that IL-6 was significant on day 3 (OR 2.6) with an AUC/ROC of 0.86. Contrary to our study, Pettila et al. (18) found in 61 critically ill patients with suspected sepsis a trend of IL-6 increase (1000 pg/mL – day 1 and 2000 pg/mL – day 2) in non-survivors; but in survivors the trend was the same as in our study (426 pg/mL – day 1, 162 pg/mL – day 2). In this study, IL-6 showed good discriminative power in the prediction of hospital mortality only on day 2 with an AUC (95% CI) of 0.79. There are several studies in which the daily trend of inflammatory mediators was not assessed, but their predictive capacity regarding outcome was based on one sample taken at the time of patient inclusion in the study. In two of them (19, 20), IL-6 showed moderate predictive value regarding mortality in critically ill septic patients with an AUC (95% CI) between 0.67 and 0.71. Contrary to that, Cheval et al. found that IL-6 was neither a predictor of infection nor 28-day mortality in 60 critically ill patients, as we did in our study (21). It seems that IL-6 did not meet the high expectations regarding predictive value. One possible explanation is that this cytokine may occur in relative asynchrony with other mediators and/or clinical manifestations in the critically ill.

In our study, there was a difference in the daily trend of CRP between survivors and non-survivors but it did not reach statistical significance. CRP was not a reliable predictor of outcome. Overall, mean values were higher in survivors except for day 2. In the study of Miguel-Bayarri et al. (17) there was a somewhat different trend. CRP was higher in survivors on day 1, lower on day 3 and statistically significantly lower on day 7. Similar to our results, Pettila et al. (18) found higher CRP values in survivors on day 1 and in non-survivors on day 2; the difference was not statistically significant, and the predictive value regarding outcome was low with the AUC (95% CI) 0.38 and 0.53 respectively. Contrary to our results, Devran et al. (22) found that CRP was not a good predictor of outcome on day 1 (AUC 0.57) but its discriminative power in prediction of mortality increased on day 3 with an AUC of 0.72 and they concluded that CRP is a valuable predictor of mortality in patients with severe sepsis due to...
physiological acute inflammatory or innate immune response. CD64 appears to be a marker of neutrophil activation or systemic acute inflammatory response as its expression starts from less than 1000 to 2000 sites per cell (resting state) and becomes up-regulated in a graded fashion depending upon the intensity of stimulation (within 4 to 6 hours it can reach more than 10-fold higher levels) contrary to monocytes with a constitutively expressed CD64 antigen (29). Neutrophil CD64 index is designed so that normal inactivated neutrophils yield values of < 1.00 and blood samples from individuals with documented infection or sepsis typically show values > 1.50.

Of all the biomarkers investigated in our study, CD64 index showed the best performance regarding mortality prediction. There was a statistically significant difference in CD64 index between survivors and non-survivors on day 1 and day 2 of the study. CD64 index was 1.6-fold higher on day 1 and 1.78-fold higher on day 2 in non-survivors. Receiver operating curves were generated to determine cut-off values for optimal sensitivity and specificity for the CD64 index levels on day 1 for outcome (Figure 1). The area under the curve (AUC) for the CD64 index on day 1 plots for outcome was 0.727; p<0.05 (Figure 1). We concluded that CD64 index on day 1 has good discriminative power in the prediction of hospital mortality. At a cut-off level of 2.80, sensitivity was 75% and specificity was 65%. Patients with CD64 index level on day 1 higher than 2.80 had 2.4-fold higher probability of death than those with lower values. Odds ratio is 2.40; 95% CI 0.60–9.67.

Several studies focused on neutrophil CD64 and outcome in critically ill patients concur with our results. Hsu et al. enrolled 66 critically ill patients in their study (30) and they showed that CD64 expression was significantly higher in non-survivors than in survivors and that this biomarker was a significant predictor of mortality with an AUC of 0.70. Disseminated intravascular coagulation (DIC) is frequent in critically ill patients. Song et al. investigated the association of neutrophil CD64 expression with severity and prognosis of DIC (31). They enrolled 94 patients with suspected DIC and their results demonstrated that neutrophil CD64 expression was significantly increased in a 28-day non-survival group and that the 28-day survival rate showed a stronger association with the neutrophil CD64 expression than with the DIC score. Prognostic value of this biomarker in terms of 28-day mortality was very good with an AUC of 0.81. In another study, authors investigated neutrophil CD64 expression as an early marker of severity and outcome in 47 critically ill septic patients (32). They found that CD64 expression was associated with the 28-day mortality (OR=1.3, p=0.01); ROC curve analysis showed that CD64 was a good predictor of outcome with an AUC of 0.75. In their patient population, an increase of CD64 expression on neutrophils by 1000 units increases 1.3-fold the probability of death. Contrary to our results, some
authors showed a correlation between survival and higher CD64 expression. Cid et al. (33) included 132 patients with fever > 38 °C during the previous 24 hours from the emergency department in their study. They found that survivors showed a higher CD64 index when compared with non-survivors. ROC curve analysis for predicting outcome showed an AUC of 0.71 with the cut-off value of 1.5, with sensitivity of 85% and rather low specificity of 33%. Our patient population was significantly different than in this study. There are two studies (34, 35) with a rather small number of severe septic patients (31 and 20 respectively) demonstrating that survival is associated with increased CD64 antigen expression. In both studies the conclusion is very similar: poor outcome is due to neutrophil inactivation with reduced phagocytic activity during compensatory antiinflammatory response syndrome (CARS). But, there is growing evidence of the role of proinflammatory mediators and acute phase response in developing immune dysfunction (36, 37). This observation may contribute to the explanation of the apparent paradox of immune suppression presence in a patient with manifested hyperinflammation (38). Clinically, many patients show signs of persisting inflammation and immune-mediated organ damage while simultaneously remaining highly susceptible to secondary infections, suggesting the term complex immune dysfunction syndrome – CIDS (3). The novel investigations of sepsis point out that virtually all immune cells demonstrate immune hypoactivity. For example, neutrophils display a dual state by the concomitant presence of activation and dysfunction features. In the critically ill patients, dysfunction of organs is, to a considerable degree, driven by neutrophils, which are key immune cells (39, 40). They tend to express surface markers of activation (increased levels of CD11b and CD64), but simultaneously they display major impairment of phagocytic capacity and generation of reactive oxygen species (ROS). Mortality rate can rise above 50% in critically ill patients with immune dysfunction despite modern therapy (41), so our goal was to conduct a real-life study regarding biomarkers in an attempt to improve the standard of care for this patient population.

Conclusions

It is evident that there are conflicting findings for each of the investigated biomarkers. It might be due to the relatively small number of patients and/or slightly different patient populations. Unlike IL-6, CRP and LBP, in our study the neutrophil CD64 index showed good discriminative power in the prediction of hospital mortality. However, large multicenter studies are warranted to validate this biomarker in the routine clinical ICU setting.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

References


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