

# Chapter 6

## Longitudinal neutrophil CD64 expression as a biomarker for acute infection and severity of disease in critically ill patients.

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## **Abstract**

### Introduction

Neutrophilic granulocytes express cluster of differentiation 64 (CD64) antigen upon activation. CD64 can be used as a marker of bacterial infection and sepsis. The goal of this study was to determine whether CD64 is a useful biomarker for critically ill patients and analyze longitudinal measurements with regard to outcome and sepsis severity.

### Methods

In this prospective observational study, CD64 analysis was performed daily until discharge from ICU or death. Demographics, clinical, laboratory data, and outcome defined as 28-day survival were recorded. Patients were included when admitted to the ICU with sepsis, severe sepsis, or septic shock and within 24 h from start of antibiotic treatment.

### Results

Hundred and fifty-five consecutive patients were enrolled. At baseline, a difference in CD64 of 2.26 (1.33-4.47) vs. 1.49 (0.89-2.24) ( $P = 0.004$ ) was seen between patients with a positive culture and negative culture. CD64 at day 1 was higher with patients with septic shock when compared with sepsis ( $P = 0.012$ ). No difference of CD64 between survivors and nonsurvivors was seen.

### Conclusion

This study demonstrated that CD64 discriminates between critically ill patients with culture positive and negative sepsis and correlates with severity of disease. However, CD64 index is not a good predictor for 28-day mortality in the critically ill patient.

## **Introduction**

Sepsis remains a major cause of mortality in medical and surgical intensive care units, and delayed antibiotic therapy is strongly associated with a worse outcome.<sup>1,2</sup> Therefore, early diagnosis of sepsis is of great importance, but can be clinically challenging. Conventional clinical signs have proven not to be specific or sensitive for the diagnosis of sepsis. Laboratory parameters, like C-Reactive Protein (CRP), may increase during systemic inflammatory response syndrome (SIRS) but also in the occasion of infection. Procalcitonin (PCT) is generally considered as a biomarker that is more specific and sensitive for infection than CRP, however further research in critically ill patients is needed.<sup>3</sup> Hematological measurements such as total white blood cell count (WBC), absolute neutrophil count (ANC), immature granulocytes, band cells, immature granulocyte/total neutrophil ratio (IT ratio) are relatively uninformative if they are used in the diagnosis of sepsis. Therefore, various clinical trials have examined possible new and more reliable biomarkers over the past years to discriminate between bacterial sepsis and other causes of SIRS. One of these promising biomarkers, which gained attention over the past years, is CD64 [Cluster of Differentiation 64 antigen]. The upregulated expression of CD64 on the surface of neutrophils has been used as a marker of sepsis and predictor of outcome with varying results.<sup>4-10</sup> The role of neutrophils in sepsis is pivotal. Neutrophils are primed by bacterial products and endogenous mediators and their subsequent activation is crucial in the pathogenesis of sepsis.<sup>11</sup> CD64 is a membrane glycoprotein expressed on monocytes and macrophages. It is the high affinity neutrophil Fc $\gamma$ RI receptor which is weakly expressed by neutrophils at physiological levels, but strongly upregulated within 4-6 hours by the pleiotropic cytokines such as Interferon- $\gamma$  (IFN- $\gamma$ ) and Granulocyte Colony Stimulating Factor (G-CSF) which are produced in sepsis.<sup>3,12,13</sup> The difference in expression in resting and activated neutrophils is much higher for Fc $\gamma$ RI than for Fc $\gamma$ RII (CD32) and Fc $\gamma$ RIII (CD16), which potentially makes CD64 antigen upregulation the most useful reflection of neutrophil activation.<sup>3,14</sup> The primary purpose of this study was to determine whether this promising hematological sepsis indicator is useful as a biomarker for bacterial infection in adult critically ill patients. Secondly, since longitudinal data of CD64 expression are limited in the critically ill patient, a further goal was to clarify longitudinal expression patterns of CD64 on neutrophils with regard to outcome and sepsis severity.<sup>3</sup>

## **Materials and Methods**

### Patients

This prospective observational study was conducted between August 2011 and March 2013 in the Intensive Care Unit at the University Medical Center Utrecht, (Utrecht, the Netherlands). All patients admitted to the ICU and receiving their first dose of antibiotics within 24 hours for an assumed or proven infection before inclusion. Prescription of antibiotics was supported by microbiological evidence of infection, but a strong clinical suspicion of sepsis was also accepted for inclusion. Once

antibiotics were administered (T=0) and patients or their next of kin were asked for informed consent, the first blood sample for flow cytometry was obtained as soon as possible. Patients in need of prolonged antibiotic treatment were excluded, for instance for endocarditis, liver- or cerebral abscesses. Patients with viral, parasitic or fungal infections or immunocompromised patients were also excluded. Selective Digestive Decontamination (SDD) or Selective Oral Decontamination (SOD) was allowed, being common practice in the Netherlands.<sup>15</sup> Patients were classified on admission into three classes sepsis, severe sepsis and septic shock SIRS plus infection was defined as 'sepsis', sepsis complicated with organ dysfunction as 'severe sepsis' and 'septic shock' as sepsis with arterial hypotension despite adequate fluid resuscitation according the American College of Chest Physicians and the Society of Critical Care Medicine Consensus Conference (Northbrook, IL, USA; August, 1991).<sup>16</sup> Longitudinal analysis was conducted, examining the neutrophil surface expression of CD64, clinical signs of sepsis and severity of disease on a daily basis until discharge from ICU or death. This protocol was approved by the appropriate ethics committee, the institutional review board and has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Informed consent from patients or their next of kin had to be obtained prior to their inclusion in the study.

#### CD64 Index

Expression of FcγRI (CD64) on the neutrophil surface was measured within 4 hours after phlebotomy in EDTA anticoagulated whole blood without prior manipulation using direct immunofluorescence on a Cell-Dyn Sapphire hematology analyzer (Abbott Diagnostics, Santa Clara, Ca, USA). Blood samples were prepared according to the manufacturer's instructions; 100 μL of whole blood was mixed with 50 μL antibody solution containing FITC-labeled CD64 (clones 22 and 32.2) and PE-labeled monocyte specific CD163 antibodies and 20 μL fluorescent beads for calibration and standardization. Prior to analysis, the samples were incubated at room temperature for 10 min. The cell populations were identified by their scatter profiles, and monocytes by their CD163 expression. Monocytes and lymphocytes were used as internal positive and negative controls, respectively, and the fluorescent beads were used for calibration. Dedicated software was used for automated calculation of the neutrophil CD64 index (Leuko64; Trillium Diagnostics, Brewer, ME, USA). All expression data was obtained as expression indices calculated by the software (denoted CD64 index), corresponding to the mean receptor density per cell. The PMN CD64 index is supposed to be less than 1.00 in healthy individuals.<sup>17</sup> The hematology analyzer uses spectrophotometry, electrical impedance, laser light scattering (multi angle polarized scatter separation (MAPPS)), and 3 color fluorescent technologies to classify blood cells.<sup>18</sup> Moreover, the analyzer is equipped with an integrated fluorescence (488 blue diode) laser and three fluorescent detectors, which allows use as a flow-cytometer in a routine laboratory setting.<sup>19</sup> The results are reported as a PMN CD64 index. The measurements were performed by experienced technicians in the central laboratory, who were blinded to the clinical data.

### Data acquisition

For each patient the following data were collected: age, gender, main reason for admission, infection diagnosis, stratification into sepsis, severe sepsis and septic shock, APACHE IV score, sepsis-related organ failure assessment (SOFA), mortality in the ICU and routine laboratory results, like C-reactive Protein (CRP) was performed according to standard of care. CRP was measured using a fully automated DxC clinical chemistry analyzer (Beckman-Coulter, Brea, CA, USA); The reference range for CRP was established at < 10 mg/L. Daily samples were used for CD64 flow cytometry assays. To confirm a bacterial infection, all necessary cultures were performed on admission. The following samples for culture were obtained, dependent on the suspected infection site, urine, respiratory tract samples (sputum, trachea aspirates, broncho-alveolar lavages) blood in at least two pairs of hemoculture bottles per patient. Standard microbiology methods on agar plates identified pathogens when the growth was positive. All potential pathogenic micro-organisms were considered as positive cultures.

### Statistical analyses

Statistical analyses were performed using SPSS version 20. Continuous data were summarized as means (standard deviation) for normally distributed data and median (interquartile range IQR) otherwise. Normality of distributions was tested using the Kolmogorov-Smirnov test. Categorical variables were summarized by number (percentages) of patients per category. Associations between continuous variables were quantified using Spearman's correlation. Continuous variables were compared between groups using Mann-Whitney U tests (two groups) or Kruskal Wallis ANOVA (more than two groups). In case of a significant Kruskal Wallis ANOVA, posthoc comparisons of outcomes between each pair of groups were performed using Mann-Whitney U tests applying a Bonferroni correction to account for multiple testing. Longitudinal course of CD64 over time was compared between groups using mixed models. Models included a main effect of days since inclusion (a linear and quadratic term), an indicator for group and their interaction. CD64 measurements were log-transformed in order to satisfy the normality assumption for the residuals that is required in mixed model analysis. Corrections for age, apache score, gender and intervention group were performed by adding main-effects for these variables in the mixed models, together with their interaction a linear and quadratic term for days since inclusion. For the purpose of plotting predicted values log-transformed CD64 were transformed back to the original scale. A two-sided p value of less than 0.05 was deemed statistically significant.

### **Results**

From August 2011 until March 2013, 1096 consecutive patients were screened and 941 patients were excluded based on not meeting the criteria for enrollment like receiving antibiotics as prophylactic therapy (n=642), receiving antibiotics longer than 24 hours before inclusion (n=124), in need for long-

term antibiotics (n=46), declined or withdrawn informed consent (n=22) Finally, 155 patients were included and serial CD64 measurements were performed. All patients received their first dose of antibiotics within 24 hours for an assumed or proven infection before inclusion.

<b>Table 1 Patient characteristics</b>				
<b>Characteristics</b>		<b>CD64 day 1</b>	<b>P-value</b>	<b><math>r_s</math></b>
	n= 111	1.64 (1.08-3.29)		
<b>Gender</b>			0.29	
<b>male</b>	75 (68)	1.51 (1.00-3.15)		
<b>Female</b>	36 (32)	1.87 (1.17-3.49)		
<b>Apache IV</b>	77 (60-92)			
<b>ICU mortality within 28 days</b>			0.70	
<b>Survivors</b>	83 (74)	1.51 (1.11-3.3)		
<b>Non-survivors</b>	28 (25)	1.81 (0.92-3.28)		
<b>Stratification at time of inclusion</b>			<b>0.005</b>	
<b>Sepsis</b>	68 (61)	1.48 (0.93-2.17)		
<b>Severe sepsis</b>	17 (15)	1.93 (1.39-4.22)		
<b>Septic shock</b>	26 (23)	2.89 (1.41-5.07)		
<b>Source at inclusion of study</b>			0.33	
<b>Hospital-acquired infection</b>	34 (31)	1.57 (0.86-2.80)		
<b>Community acquired</b>	77 (69)	1.74 (1.11-3.58)		
<b>Microbiological culture on admission</b>			<b>0.004</b>	
<b>Positive Culture</b>	42 (38)	2.26 (1.33-4.47)		
<b>Negative Culture</b>	69 (62)	1.49 (0.89-2.24)		
<b>Culture on admission</b>			0.71	
<b>Gram-negative</b>	25 (23)	1.74 (1.22-4.03)		
<b>Gram-positive</b>	19 (17)	2.72 (1.42-4.90)		
<b>Laboratory variables</b>				
<b>CRP</b>	179 (82-270)		<b>&lt;0.001</b>	<b>0.46</b>
<b>White blood cells</b>	13.5 (9.1-20.0)		0.77	<b>0.03</b>
<b>Thrombocytes</b>	213 (153-296)		<b>0.02</b>	<b>-0.22</b>
<b>Creatinine</b>	84 (59-131)		<b>0.05</b>	<b>0.18</b>

Analysed by Mann-Whitney U test, Kruskal Wallis H-test and Spearman correlation (  $r_s$  )

Data are expressed as number (percentage), median and IQ-quartiles

### CD64 index at day 1 and its association with severity, patient characteristics and other markers

A CD64 measurement was performed at the same day of inclusion (day 1) in 111 out of 155 patients (72%). The median CD64 index at day 1 was 1.64 (IQR:1.08-3.29) with a mean of 2.34. Medians and IQR for CD64 at day 1 are tabulated per group and compared in Table 1.

Sixty-one percent of all patients were stratified as septic, 15 % as severe septic and 23 % as septic shock. CD64 measurements at day 1 were found to differ between these three categories ( $p$  Kruskal Wallis ANOVA = 0.005). Posthoc tests revealed CD64 measurements at the first day to be higher with septic shock when compared to patients with sepsis (Bonferroni corrected  $p$  Mann Whitney test = 0.012), but no differences were found between the sepsis and severe sepsis ( $p$  = 0.096) and between severe sepsis and septic shock ( $p$  = 1.00). CD64 index showed a positive association with CRP (standard beta coefficient = 0.46;  $p$  <0.001), thrombocytes (-0.22,  $p$  = 0.02) and serum creatinine (0.18,  $p$  = 0.05). No difference in CD64 index on day 1 was seen with regard to gender, between survivors and non-survivors (AUC = 0.52, 95% CI [0.40, 0.65],  $p$  = 0.70) and hospital versus community-acquired infections (Table 1). Patients were divided between patients with a positive culture during the first 24 hours of inclusion. Cultures of sputum, blood, peritoneal lavage, bronchial lavage, and urine were evaluated. Cultures from nasal- and throat swabs, which were merely performed for surveillance purposes (as demanded by SDD or SOD protocols), were not considered as positive culture. The baseline CD64 index at day 1 was significantly higher in patients with a positive culture, 2.26 (1.33-4.47) versus 1.49 (0.89-2.24) for the patients in whom no micro-organisms were cultured ( $p$ =0.004) (Table 1). No difference was seen when sub-analysis was performed for gram-negative versus gram-positive bacteria. Positive cultures with *E. coli* showed the highest mean of CD64 measurements (figure A; supplementary material).

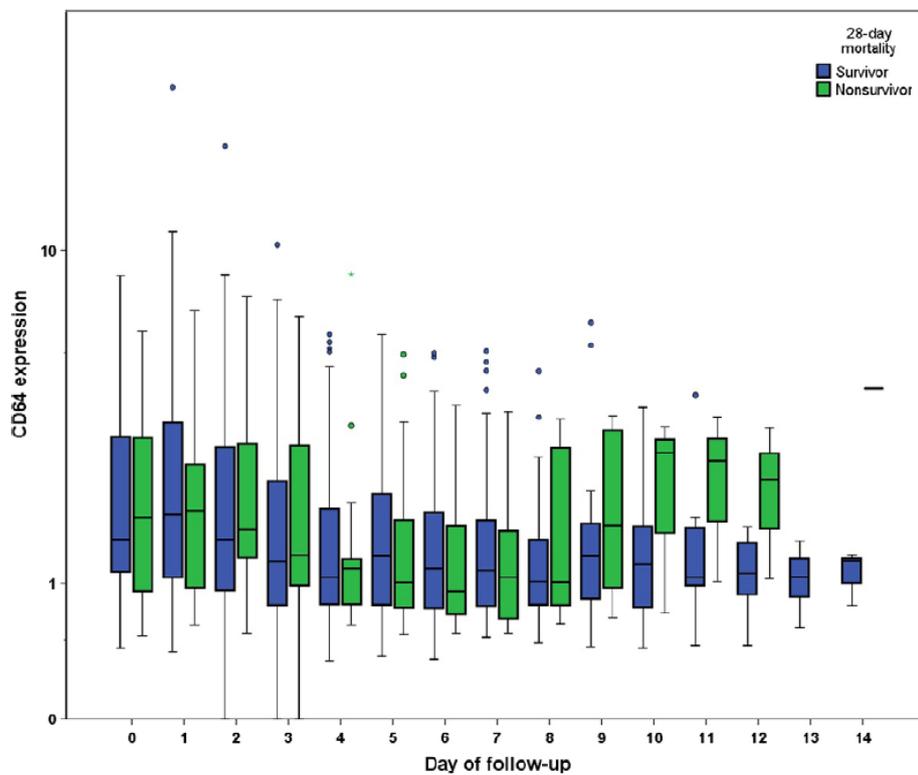
### Longitudinal course of CD64 index as a function of positive cultures, survival and severity of disease

Longitudinal courses were analyzed in all 155 patients. Uncorrected analysis showed a significant difference in the longitudinal course of CD64 index during the first 14 days of follow-up with  $p$ -value for the quadratic term of the interaction being  $p$  = 0.192, but a significant linear part after removal of the quadratic term ( $p$ =0.001) indicating a stronger decrease in the positive culture group. This linear term remained significant after correction for intervention group, age, gender and apache-score ( $p$ =0.006).

### Measurement of CD64 index as a function of survival

The overall mortality within 28 days was 21%. The maximum follow-up for CD64 measurements was restricted to 14 days. No differences were seen in duration of follow-up between survivors and non-survivors ( $p$  = 0.44). Uncorrected analysis showed no significant difference in the longitudinal course of CD64 index during the first 14 days of follow-up between survivors versus non-survivors. The  $p$ -value for the quadratic term of the interaction between group (survivors/non-survivors) and time of

CD64 measurement was 0.65. After removal of the quadratic term of the interaction, the linear term had a p-value of 0.08. When we corrected for intervention group (including interactions between intervention group and linear and quadratic terms for time of measurement), the quadratic term was still not significant (p=0.49). After removal of the quadratic term of the interaction with intervention, the linear term became significant (p = 0.016) indicating a stronger decrease in mean CD64 index over time in the survivor group. The linear term of interaction between time and survival group became even stronger when we in addition corrected for age, gender and apache-score (p = 0.003). These longitudinal course of mean CD64 index are depicted graphically in figure 1.

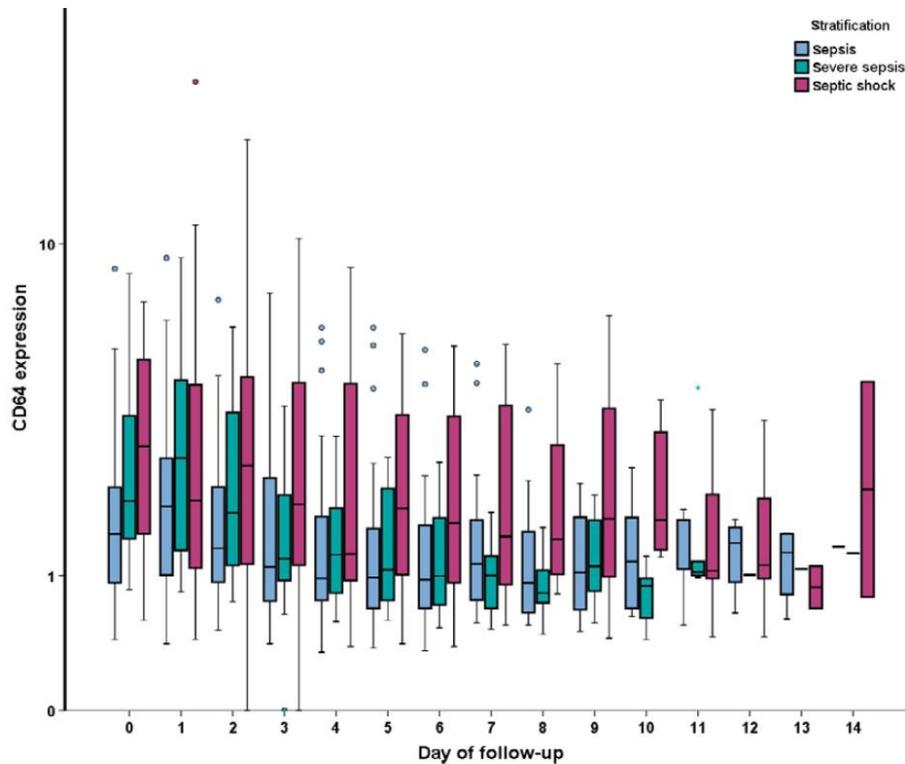


**Figure 1** Longitudinal course of mean CD64 in survivors and non-survivors

Measurement of CD64 index as a function of severity of disease

Longitudinal course of mean CD64 for patients with sepsis, severe sepsis and septic shock during the 14 day period are presented graphically in figure 2. A difference was seen in the course of CD64 index between patients with sepsis, severe sepsis and septic shock. The p-value for the quadratic term of the interaction between group and days was 0.005 and the linear term had a p-value of 0.026. Both terms were no longer significant, when we corrected for intervention group, age, gender and apache-score.

After correction, only the main effect for patient group was found to be significant indicating differences in mean CD64 index at day 1 (conform results mentioned at beginning of this section).



**Figure 2** Longitudinal course of mean CD64 in groups with different severity of sepsis

## Discussion

Previous studies have shown that CD64 expression on neutrophils is significantly increased in patients with sepsis.<sup>8,20-24</sup> We confirmed these findings and in addition we demonstrated that CD64 expression on neutrophils strongly correlates with severity of sepsis. However, in conflict with the study of Chen et al. our study CD64 index is not a good predictor of mortality.<sup>22</sup> This prospective observational study is of particular interest because of the longitudinal analysis of the CD64 expression in a large cohort of ICU patients. Consecutive ICU patients were enrolled early, all with strong clinical suspicion of sepsis and all patients received their first dosage of antibiotics within 24 hours before enrolment. Two previous studies also performed longitudinal analysis of neutrophil CD64 expression.<sup>3,24</sup> However, there are some differences in their longitudinal patterns of CD64 expression in comparison to our observations. Our study showed an increase after the first day for all patients. This may reflect an early upregulation of neutrophil activation caused by acute infection. This early CD64 rise was followed by a decline of CD64 expression after day 3, which might be an indirect effect of antibiotics causing restoration of the regulated immune response.<sup>25</sup> Alternatively, the decline in CD64 might also represent a state of neutrophil deactivation with a decreased PMN phagocytic function and subsequent

lower levels of CD64 expression, which has been previously described in patients with severe sepsis.<sup>26</sup> Or, the decline in CD64 expression may have been the result of the so-called Counter Anti-inflammatory Response Syndrome (CARS), which is considered as an adapted compartmentalized response with the main aim to silence some acute pro-inflammatory genes while maintaining the possible expression of certain genes involved in the anti-infectious process.<sup>27</sup> All of the included patients received early antibiotics before inclusion to the study, which might explain the early decline after one day of CD64-expression in all three groups. In this regard our study is markedly different from the previously mentioned study of Fisher et al.<sup>3</sup> Considering the time course of CD64 index it would be interesting to investigate whether daily monitoring of CD64 expression during antibiotic therapy can better inform decisions to discontinue antibiotics, analogous to serial procalcitonin-guided studies.<sup>28</sup> However, further research is needed to investigate the best cut-off point for a suitable recommendation on discontinuation.

Secondly, this study demonstrates that an early rise (in the first 2 days) in the CD64 index is capable of discriminating between relevant positive and negative cultures, consistent with the study by Cardelli et al., who retrospectively showed neutrophil CD64 expression was higher in patient with positive blood cultures. Our study included all positive cultures (such as blood, tracheal aspirates and peritoneal lavages).<sup>5</sup> Previous findings indicating that infections caused by Gram-negative bacteria had higher levels of CD64 expression compared with the Gram-positive infections were not confirmed by our results.<sup>12,20,27</sup> In fact our work supports more recent studies, where no significant difference in CD64 expression was seen between gram-negative and positive infections.<sup>17,30-32</sup>

Another important finding of our study is that the neutrophil CD64 expression is an indicator for severity of disease, which is consistent with the study by Livaditi et al. who showed that neutrophil CD64 expression, measured only at the onset of sepsis, correlated strongly with the severity of disease.<sup>6</sup> They also found high CD64 expression to be a good predictor for mortality, in contrast to our findings. No difference was found in CD64 expression between survivors and non-survivors in the first 28 days of follow up.

In our study we were able to measure the CD64 index 24/7 using the routine hematology analyzer. However, in many laboratories this flow cytometric analytic capability is not available on a 24 hour basis.<sup>13</sup> Again, this might have influenced the results and might explain some of the differences with previously published studies. Some limitations to this study should be noted. Firstly, the significance of clinical sepsis with culture negative patients remains debatable.<sup>33</sup> Patients were included whenever they were suspected of having clinical infection and were administered antibiotics. However, 60% of all patients remained culture negative. Obviously, some of those patients had false negative cultures while others were merely experiencing SIRS and no sepsis. These patients were combined in the “culture negative” group. Secondly, the number of patients, especially in the severe sepsis and septic shock group were relatively small, extrapolation of these results should be done with care. A larger

number is needed for more precise evaluation in critically ill patients and for subgroup analysis. Third, for this study there was no predefined control group.

### **Conclusion**

We have demonstrated a clear difference in CD64 expression on days 1 and 2 between septic patients with subsequent positive and negative cultures. We have also shown that CD64 expression correlates with disease severity as expressed by SOFA or severity of sepsis. In contrast to other studies, we have found no prognostic relation between CD64 and mortality. The assay is relatively simple to perform and with the right equipment can be available 24 hours a day. This can be of additional value, next to the current tests, in anticipation of the results of the culture, which remains the golden standard. However, further studies are needed to expand the potential utility of CD64 as a novel biomarker for diagnosing and/or monitoring sepsis.

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On behalf of all authors, the corresponding author states that there is no conflict of interest.

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