

EVALUATION OF NEUTROPHIL CD64 EXPRESSION AND PROCALCITONIN AS USEFUL MARKERS IN EARLY DIAGNOSIS OF SEPSIS

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Quantitation of neutrophil CD64 expression and procalcitonin (PCT) levels in blood samples have been recently proposed as useful tools for early detection of sepsis. To determine the usefulness of these tests, we analyzed blood samples of 112 patients, admitted to an intensive care unit (ICU), presenting clinical symptoms of sepsis, as well as of 50 healthy controls. At the end of the study, a retrospective analysis showed that only 52 of the 112 ICU-patients presented a real sepsis (positive blood culture). The results obtained indicated that of the 52 patients with sepsis, 50 and 49 presented levels of neutrophil CD64 expression ≥ 2398 molecules per cell (cut-off determined by receiver operator characteristic analysis) and PCT levels > 0.5 ng/ml (cut-off suggested by the manufacturer), respectively. However, the neutrophil CD64 test showed higher specificity in detecting sepsis since 5 out of the 60 ICU-patients without sepsis (negative blood culture), presented CD64 expression levels ≥ 2398 molecules per cell, PCT levels ≥ 0.5 ng/ml were shown in 27 patients. Moreover, while none of the 50 healthy controls presented a neutrophil CD64 level higher than the cut-off value, 5 patients presented PCT levels ≥ 0.5 ng/ml. In conclusion, our data seem to indicate that the quantitation of CD64 expression could be taken into consideration as a sensitive and specific test for early diagnosis of sepsis.

Early diagnosis of sepsis in patients admitted to intensive care units (ICUs) is of great clinical importance (1). Conversely, diagnosis of sepsis by the conventional microbiological blood-culture technique is cumbersome, and common laboratory tests, including leukocyte counts, C reactive protein (CRP) and erythrocyte sedimentation rate (ESR), have been shown to present low sensitivity and specificity (2). Rapid highly-sensitive and -specific

laboratory tests are thus needed to improve the care of these patients. In this respect, determination of the level of blood procalcitonin (PCT) has recently gained attention (3). PCT, which is produced in C-cells of the thyroid gland, is the precursor of calcitonin which is normally cleaved by a specific protease to calcitonin and kalcalcin (4). In healthy humans, PCT is not generally released into the blood stream, while during systemic infections PCT levels

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may increase to over 100 ng/ml (5). Unfortunately, a variety of unrelated factors, including severe systemic inflammation, organ dysfunction, tissue trauma, and many other aetiologies, have been shown to induce PCT release (5-14).

The quantitation of neutrophil CD64 expression is another test which has been recently described (15-17). CD64 is a high-affinity antibody receptor involved in the process of phagocytosis and intracellular killing of microorganisms. CD64 molecules are normally expressed at a very low level on the surface of unstimulated neutrophils while their expression markedly increases in response to systemic infections (16-17). This feature renders the quantitation of neutrophil CD64 expression a potentially useful marker to detect sepsis.

In the present study we determined neutrophil CD64 expression and PCT levels in blood samples of patients admitted to the ICU of the Azienda Ospedaliera "Sant'Andrea", of the Sapienza University of Rome. Quantitation of neutrophil CD64 expression was detected by flow cytometric analysis, and cases of positive sepsis were confirmed using classical blood-culture techniques. The usefulness and performance statistics (sensitivity, specificity, positive and negative predictive values) of both tests in detecting systemic infections have also been calculated.

MATERIALS AND METHODS

Selection of patients and controls

112 patients (53 female and 59 male; mean age 63 ± 9.7) admitted to the intensive care unit of the Azienda Ospedaliera "Sant'Andrea" (II° Facoltà di Medicina e Chirurgia) University Sapienza, Rome, Italy from January 2005 to December 2005 were enrolled in this study. Sepsis (clinical sepsis) was suspected, as defined by the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM), only when patients presented at least two of the following symptoms: fever or hypothermia ($>38^\circ\text{C}$ and $<36^\circ\text{C}$), tachycardia (>90 beats/min), tachypnea (>20 breaths/min) or $\text{PoCO}_2 < 32$ mmHg and leukocytosis ($>12000/\mu\text{l}$), leukopenia ($<4000/\mu\text{l}$), or more than 10% immature forms of white blood cells (18). Blood samples of patients were taken within the first 6 hours of sepsis being suspected, and neutrophil CD64 expression and PCT levels were determined. Sepsis was confirmed by blood-culture analysis. Urine samples, bronchoalveolar lavages, sputum, surgical swabs, and

catheters were also taken for microbiological examinations before starting antibiotic therapy was. Moreover, blood samples of 50 healthy controls (27 female and 23 male; mean age 58 ± 11.4), randomly selected from patients attending the routine clinical haematology laboratory, were also included in this study.

Flow cytometry

Neutrophil CD64 expression was measured by staining 50 μl of whole blood with a 20 μl mixture of anti-CD64-PE and anti-CD45-PerCP (Becton-Dickinson, San Jose, CA) for 1 hour at room temperature, in the dark. At the end of the incubation, red blood cells were lysed by the addition of 2 ml of 1x FACS Lysing solution (Becton-Dickinson). After incubation for an additional 1 hour at room temperature, specimens were analysed on a FACSCalibur flow cytometer with CellQuest software for quantization analysis (Becton-Dickinson, San Jose, CA). By evaluating the CD45/side scattering profiles, lymphocytes, monocytes and neutrophils were identified and gated. At this point, a calibration curve was constructed using QuantiBRITE PE beads (Becton Dickinson), as suggested by the manufacturer, to quantify neutrophil CD64 expression. The calibration curve was imported into the CellQuest software. Ten thousand events were acquired for each stained blood sample and a mean fluorescent intensity (MFI) was calculated for each neutrophil cell. The number of CD64 molecules/neutrophil cell was extrapolated by comparing MFI values of the blood samples with those of the calibration curve. As there were no recommended diagnostic cut-off values for neutrophil CD64, a receiver operator characteristic (ROC) analysis was performed and neutrophil CD64 values ≥ 2398 molecules/cell were found to be suggestive of possible sepsis.

PCT

PCT levels in blood samples were measured with an immunoluminometric assay, using the BRAHMS PCT sensitive LIA kit (DASIT, Cornaredo, Milan, Italy). The kit comprises two distinct monoclonal antibodies which recognize the calcitonin or katalcalcin PCT epitopes. The anti-katalcalcin antibody is immobilized on the surface of a coated tube while the anti-calcitonin antibody, labelled with a luminescent acridine derivative compound (tracer), is in solution. In the PCT test, 50 μl aliquots of plasma were mixed with a 200 μl solution containing the tracer antibody in the coated tube and the mixture was incubated at room temperature for 2 hours. Antibodies recognition of PCT epitopes induces formation of sandwich complexes. After intensive washing, PCT was quantified by the addition of LUMitest Basiskit reagent (300 μl) and luminescence was immediately measured using a

Table I. Determination of neutrophil CD64 expression and PCT levels in blood samples of 112 ICU-patients presenting clinical symptoms of sepsis.

Assay	Cut-off ^a	Number of patients		
		with sepsis (52) ^b	without sepsis (60) ^c	controls (50) ^d
CD64	≥ 2398	50	5	0
	< 2398	2	55	50
PCT	≥ 0.5	49	27	5
	< 0.5	3	33	45

^a Neutrophil CD64 expression ≥2398 and PCT levels ≥0.5 ng/ml were used as cut-offs. The CD64 cut-off value was arbitrarily determined using ROC curve analysis while the PCT value was as suggested by the manufacturer (BRAHMS). Values are expressed as neutrophil CD64 molecules/cell and ng of PCT per ml of plasma; ^bpatients that presented a positive blood culture; ^cpatients that presented a negative blood cultures; ^dhealthy controls.

Table II. Performance statistic analysis of neutrophil CD64 expression and PCT level determinations in recognizing patients with sepsis^a.

	Neutrophil CD64	PCT
Sensitivity (%)	96	94
Specificity (%)	95	70
PPV ^b (%)	91	60
NPV ^b (%)	98	96

^aCut-off levels for neutrophil CD64 (≥ 2398) and PCT (≥ 0.5 ng/ml) were determined as described in the footnote of Table I. ^bPPV and NPV are performance statistic parameters indicating positive and negative predictive values, respectively.

Berthold LB 952 (T/16) luminometer. PCT concentrations (ng/ml of plasma) were extrapolated using a standard curve constructed with known concentrations of synthetic intact human PCT, supplied by the manufacturer. As suggested by the manufacturer of the kit, PCT values ≥0.5 were considered suggestive of possible sepsis.

Statistics

All statistical tests were performed by Prism 4 for Windows version 4.0 (GraphPad Prism). Comparison between patient groups were carried out using non-parametric test (Mann-Whitney U test). Patients with and without sepsis, as well as healthy controls, were well-

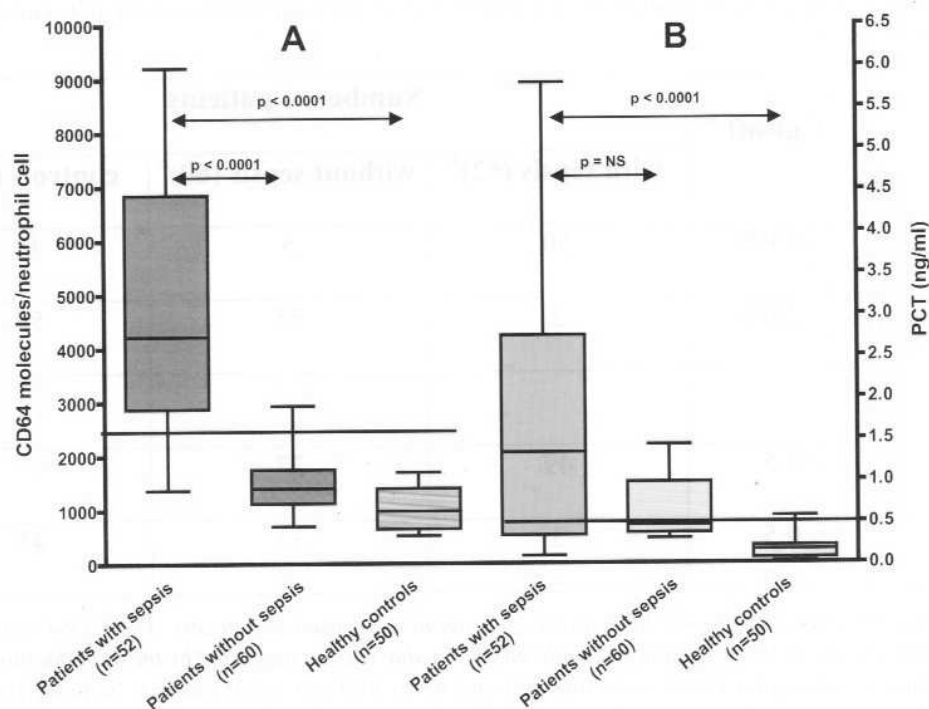


Fig. 1. Boxed representation of CD64 (A) and PCT (B) results for the three groups of patients. The line in the boxes shows the median value. The box shows the 25th–75th percentiles, while the whiskers indicate the 10th and 90th percentiles. Continuous lines represent the cut-off values. The groups are significantly different for CD64 while are not for PCT.

matched for age and sex. Receiver operator characteristic (ROC) analysis was used to establish the optimal cut-off value for neutrophil CD64 measurements. To minimize misclassification of cases of sepsis we chosen the optimal cut-off value of ≥ 2398 molecules/cell because it was approaching 100% sensitivity and presented the highest values of specificity (18). Sensitivity, specificity, positive and negative predictive values of neutrophil CD64 and PCT tests were calculated on the basis of the following formulas: $TP/(TP+FN)$; $TN/(TN+FP)$; $TP/(TP+FP)$; and $TN/(TN+FN)$, respectively. TP (true positive), stands for patients with positive blood cultures who presented neutrophil CD64 and PCT levels above the cut-off values; TN (true negative), stands for patients with negative blood cultures who presented neutrophil CD64 and PCT levels below the cut-off values; FP (false positive), stands for patients with negative blood cultures who presented neutrophil CD64 and PCT levels above the cut-off values; and FN (false negative), stands for patients with positive blood cultures who presented neutrophil CD64 and PCT levels below the cut-off values.

RESULTS

Blood-culture analysis indicated that 52 (27

female and 25 male; mean age 68 ± 4.7) out of the 112 ICU-patients examined had sepsis (they showed a positive blood-culture). *Pseudomonas aeruginosa* (14 patients), *Escherichia coli* (11 patients), *Enterobacter faecium/faecalis* (12 patients), *Staphylococcus aureus* (13 patients) and *Candida* spp. (2 patients) were the microorganisms isolated from the blood samples. Of the remaining 60 ICU-patients (26 female and 34 male; mean age 61 ± 7.7) presenting negative blood cultures, the majority showed signs of inflammation (high ESR and PCR values) or of local infections (mainly of the respiratory tract). Of these, *Stenotrophomonas maltophilia* and multi-resistant *P. aeruginosa* (11 patients), *Staphylococcus aureus* (8 patients), *E. coli* (4 patients), and *Candida* spp (1 patient) were the microorganisms isolated.

Determination of neutrophil CD64 expression

In the blood samples of the 50 healthy controls, the median CD64 molecules/neutrophil cell was 979 (IQR 645–1400) (Fig. 1). The median CD64 expression in blood samples of the 52 patients with sepsis was 4226

(IRQ 2887-6845), while in the 60 patients with negative blood-cultures the median CD64 expression was 1404 (IRQ 1126-1752) (Fig. 1).

As shown in Fig. 1, CD64 expression in the 50 healthy controls and in the 60 patients without sepsis significantly differed from that of the 52 patients with sepsis ($p < 0.0001$). Receiver operator characteristic (ROC) analysis indicated that a cut-off value of 2398 CD64 molecules/neutrophil cell (area under ROC curve 0.97; $p < 0.0001$) differentiated between patients with and without sepsis. In fact, while 50 (96%) of the 52 patients with sepsis showed CD64 expression above the cut-off value, only 5 (8%) of the 60 patients without sepsis presented CD64 levels ≥ 2398 (Table I). The sensitivity of the CD64 assay was 96% with a specificity of 95%, positive and negative predictive values (PPV and NPV) were of 91% and 98%, respectively (Table II).

Determination of PCT levels

Median PCT level in blood samples of healthy controls was 0.2 ng/ml (IQR 0.06-0.27) (Fig. 1 and Table I). PCT values raised ≥ 0.5 ng/ml (the cut-off value given by the manufacturer of the PCT kit as indicative of possible sepsis) in 49 (94%) out of the 52 patients with sepsis (median 1.34 ng/ml and IQR 0.34-2.77), while the median PCT level of the 60 patients without sepsis was 0.42 ng/ml (IQR 0.37-1) (Fig. 1 and Table I). In particular, 27 (45%) and 5 (10%) of the patients without sepsis and healthy controls, respectively, presented PCT levels higher than ≥ 0.5 ng/ml (Fig. 1 and Table I). Moreover, statistical analysis of these results indicated that PCT levels in patients with and without sepsis did not differ significantly ($p = \text{NS}$). We calculated a sensitivity of the PCT assay 94% and a specificity of 70%. Positive and negative predictive values (PPV and NPV) were of 60% and 96%, respectively (Table II).

DISCUSSION

In the present study we report the results of a one-year study conducted in the ICU of the Azienda Ospedaliera "Sant'Andrea" of the University Sapienza of Rome. We evaluated the reliability of the determinations of neutrophil CD64 expression and PCT levels in blood samples for early diagnosis of sepsis.

Septicaemia is sepsis of the bloodstream caused by bacteremia, which is the presence of bacteria in the bloodstream. Sepsis is common and also more dangerous in elderly, immunocompromised, and critically ill patients, especially those admitted to ICU. In these patients diagnosis of sepsis is of crucial importance so that an effective antibiotic therapy may be initiated as soon as possible. The early diagnosis of sepsis is a difficult task for the clinician, since there are no reliable methods to distinguish between patients who are truly infected and those that are not. The matter is far more complicated considering that negative microbiologic culture results are not always indicative of the absence of a real infection, that blood culture analysis requires a long period of incubation and, conversely, that rapid diagnosis of sepsis is often of crucial importance for the survival of patients.

Symptoms of sepsis are often related to the underlying infectious process. When the infection evolves into sepsis, the symptoms of tachycardia, tachypnea, fever, leukocytosis and/or decreased urination may occur (19-20). Consensus guidelines have recently been introduced in clinical practice to recognize clinical signs and symptoms of sepsis (PIRO and ACCP/SCCM staging systems) (19-20). Unfortunately, the diagnosis of sepsis centered solely on these criteria have been found unsatisfactory (19-21). Under these circumstances, it has become common practice to administer broad-spectrum antibiotics in suspected sepsis (clinical sepsis), often leading to unnecessary and prolonged treatments that frequently increase the duration of patient hospitalization and promote the emergence of multi-resistant bacteria (22). Nowadays, several tests based on the determination of defined blood parameters are investigated for use in the diagnosis of sepsis (23-28).

Among several markers of inflammation and sepsis, neutrophil CD64 expression and determination of blood PCT levels have been recently proposed for the diagnosis of inflammation and sepsis in different medical conditions (5-9, 11, 13, 15, 17, 27). Rapid up-regulation of neutrophil CD64 expression is a phenomenon known to be controlled by the cytokines involved in the acute inflammatory response to an infection or sepsis, while the determination of PCT blood concentrations

has been recently reported to be potentially useful in differentiating sepsis from non-infectious causes (2-3, 6-7, 12-17, 22-27, 23-28).

In the present study, we report the results of a one-year study conducted in the ICU of the Azienda Ospedaliera "Sant'Andrea", of the University Sapienza of Rome. We evaluated the reliability of the determinations of neutrophil CD64 expression and of PCT levels in blood samples for early diagnosis of sepsis. Our results clearly indicate that determinations of neutrophil CD64 expression is a sensitive (96%) and specific (95%) test (positive and negative predictive values were 91% and 98%, respectively) that might be used for early diagnosis of sepsis (Table II). We found high levels (≥ 2398) of CD64 receptors expressed on the surface of neutrophils of the great majority (50 out of 52) of patients with sepsis (patients presenting a positive blood culture), while lower CD64 expression was found on blood samples of patients without sepsis (negative blood culture) and healthy controls (Fig. 1 and Table I).

Dosage of PCT levels in blood samples has been considered predictive of sepsis and a specific immunologic test kit (BRAHMS PCT sensitive LIA kit) has been recently commercialized. As recommended by the manufacturer (DASIT), PCT levels ≥ 0.5 ng/ml are proposed as indicative of possible sepsis. Under these conditions, the PCT assay was found sensitive (94%) but not as specific (70%) as the neutrophil CD64 assay (95%) for early diagnosis of sepsis (positive and negative predictive values were 60% and 96%, respectively) (Table II). In fact, PCT levels ≥ 0.5 ng/ml were found in 49 out of the 52 patients with sepsis, in 27 out of the 60 patients without sepsis and in 5 out of the 50 healthy controls (Fig. 1 and Table I).

These results are in agreement with previous reports indicating low specificity of PCT level determinations in predicting systemic infections (9-14, 20). A variety of clinical conditions have been shown to influence PCT blood levels, including the site of the infection and the presence of pathologies not strictly infection-related (severe trauma, myocardial infarction after cardiac surgery, cardiogenic shock, severe burn injury and heatstroke) (9-14, 21).

Although further studies are certainly needed, taken together our results clearly indicate that measurements of CD64 expression could be a promising tool, useful

for the early diagnosis of sepsis.

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