# Flow Cytometric Determination of Neutrophil CD64 (nCD64) in Children with Community Acquired Pneumonia

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## Abstract

### Background
The expression of CD64 in neutrophils (nCD64) has shown utility in the diagnosis of sepsis. The aim of this study was to evaluate the diagnostic value of neutrophil CD64 in pneumonia as an early marker for infection and correlate its level with the outcome of pneumonia.

### Materials and Methods
Forty children diagnosed as pneumonia and admitted at pediatric department, Minia University Children's Hospital, Egypt, were included in the study and another forty apparently healthy children served as controls. Both patients and controls were subjected to complete blood counts (CBC), quantitative C-reactive protein (CRP), arterial blood gases (ABG), and determination of CD64 expression on neutrophils by flow cytometry.

### Results
The absolute count of nCD64 in patients with community acquired pneumonia was (1140±109.7) (RFU), significantly higher than in controls (327.3± 25.7) (RFU) (p=0.001). Significant positive correlations were found between CD64 and CRP (p=0.001, r=0.76) and total leucocytic count of patients (p=0.01, r=0.76). Significant negative correlations were present between CD64 and platelet count (p=0.008, r= - 0.7), age (p=0.001, r= - 0.9) and weight (p=0.01, r= - 0.8), respectively.

### Conclusion
Determination of peripheral blood neutrophil CD64 is sensitive and specific for early diagnosis of pneumonia and high level is associated with clinical deterioration and poor outcomes.

*Key Words:* Children, C-reactive protein, CD64 in neutrophils, Pneumonia.

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## 1- INTRODUCTION

Community-acquired pneumonia (CAP) is one of the most common infectious diseases and an important cause of death in children under 5 years old in developing countries and in adults over 65 in developed countries (1). Pneumonia can result from the effect of a respiratory virus on the lungs that leads to both primary viral pneumonia and pneumonia with a secondary bacterial etiology, as well as to later bacterial complications of the respiratory tract viral illness. Some patients develop a mixed infection with a viral–bacterial etiology. In addition, CAP can be caused by several pathogens simultaneously (2). Despite the improvements in different medical aspects the mortality of CAP remains high (3).

Most of these deaths occur in the first 48 hours, when the impact of antibiotics appears to be limited (4). The high mortality rate is due to a disproportionate host inflammatory response to the microorganism and therefore early identification of patients at risk of a poor outcome seems crucial for proper clinical management and better outcomes (5, 6).

Children have an immature immune system and poor immune function, which is liable to induce a variety of infectious diseases and allergic diseases (7). No clinical features exist that allow intracellular and extracellular pathogens in pneumonia to be discerned, although extra pulmonary manifestations are often associated with intracellular pathogens in CAP. Cluster of differentiation 64 (CD64), is the high-affinity immunoglobin fragment crystallization (FC) gamma receptor (FcγR). It is expressed on cells of myeloid lineage, playing an important role in phagocytosis, clearance of immune complexes, antigen presentation, and cytokine release. Also, it is considered an important indicator of immune response when it is activated by pathogen or allergen, with a difference in the increase in the level of nCD64 according to the cause of activation (8, 9). CD64 expression on neutrophils (nCD64), increases in response to infection and returns to normal once infection disappears (10, 11). nCD64 expression is relatively stable in blood for more than 30 h. Assessment of nCD64 requires a small blood volume, and the assay method is accurate, fast, and simple (12, 13). Several studies investigated its role in the diagnosis of bacterial infection and sepsis (14-17). However, its role in diagnosis of pneumonia has not yet been fully investigated. The aim of this study was to evaluate the diagnostic value of neutrophil CD64 in children with community acquired pneumonia as an early marker of infection and correlate its level with the outcome of pneumonia.

## 2- MATERIALS AND METHODS

### 2-1. Method

In prospective case-control study which included eighty children who attended Minia University Children's Hospital, Egypt, during the period from January 2016 to January 2018. Their age of patients ranged from 6 to 60 months and they were classified into two groups: Group 1) 40 children with clinical and radiological evidence of pneumonia, Group 2) 40 apparently healthy children who attended for non-respiratory problems or serious medical condition. Any children below 6 months and/or above 60 months, children with chronic respiratory illness, dehydration, any suspicion of central nervous system (CNS) disorder were excluded from the study. The study was reviewed and approved by the Scientific Ethical Committee of the Faculty of Medicine Minia University (ID-code: 193:4/2016). Informed written consents were documented from all children's guardians. All patients and controls were subjected to full history taking, thorough clinical examination and radiological imaging by chest X-ray for patients only.

Children with pneumonia and controls were subjected to complete blood counts (CBC), quantitative C-reactive protein (CRP), arterial blood gases (ABG), and determination of CD64 expression by flow cytometry. Respiratory severity score was calculated for each patient. Patients with a score of 0-1 were considered mild pneumonia, 2-3 moderate pneumonia, while patients with a score of 4-5 were considered severe pneumonia (18).

## 2-2. Sampling protocol

Three ml of venous blood was collected by venipuncture and was divided into 1 ml on EDTA tube for CBC and flow cytometric assessment of CD64 on neutrophils, 2 ml of blood on plain tube, allowed to clot and the serum was separated by centrifugation at 3000 rpm for assessment of high sensitive CRP, and arterial blood sample was collected on heparinized tube for measurement of arterial blood gases.

## 2-3. Assay protocol

CBC by using automated cell count, Sysmex KX-21-N (TAO Medical Inc., Japan).High sensitive CRP was performed by EIA and neutrophil CD64 was assessed using flow cytometry, BD FACS Canto LS USA, the kits were supplied by R&D SYSTEMS (19).

## 2-4. Assessment of CD64 on neutrophils by Flow Cytometry

Whole blood was collected in evacuated tubes containing EDTA. For each sample, two tubes were prepared and labeled 1&2 (Control and Test).100 μL of blood sample was added to the tubes.10 μL of Anti CD64-PE FITC conjugated antibody and Anti CD45-per CP were added (only to the TEST tube), and vortexed. Cells were incubated for 15-20 min at room temperature in the dark. Cells were washed in phosphate buffered saline (PBS) twice to remove any unbound antibodies. Followed by red cell lysis using 2 ml of lysing solution then were incubated for 10

min at room temperature in the dark. Cells were centrifuged for 5 min, supernatant was discarded and 2 ml of PBS was added. Washing by PBS was repeated twice then the cells were re-suspended in 400μL of PBS for final flow cytometric analysis.

## 2-5. Analysis of data

Analysis was carried out using a (BD-FACS FLOW Argon laser USA) flow cytometry at 515nm. Data processing was carried out with the XL software.

## 2-6. Statistical methods

The collected data were analyzed using statistical package for social science (SPSS) software for windows (version 18.0.). All data were expressed as mean value ± standard deviation (SD). Comparisons of parameters among groups were made using paired t test. Comparisons between two qualitative variables were performed using Chi-square and fisher's exact tests. P value ≤ 0.05 was considered significant. Pearson's correlation coefficient (r) test was used for correlating data. Receiver Operating Characteristic (ROC) curve analysis was used to find the overall predictively of parameter and the best cut-off value with detection of sensitivity and specificity.

## 3- RESULT

### 3-1. Patient characteristics

Forty children with CAP and 40 healthy children were included in the study. The mean age in CAP and healthy children was 40.6±8.6 and 20.5±16.1 months, respectively and 57.5 % (n=23), and 70% (n=28) of CAP and healthy children were females, respectively (P>0.05). In pneumonic patients there were significant higher vital signs (heart rate, respiratory rate and temperature) compared to controls (p= 0.01, 0.01, 0.02, respectively). Regarding the clinical presentation of patients with CAP 15 patients (37.5 %) were presented with

lobar pneumonia, 18 patients (45%) with bronchopneumonia, and 7 patients (17.5%) with interstitial pneumonia. Ten cases (25%) developed respiratory failure on arrival at the emergency department and required ICU admission and needed invasive mechanical ventilation. Four (10%) patients died. Also, regarding laboratory results, there was a significant increased total leucocytic count and CRP (p= 0.001 for both), and significant decrease in O2 saturation and PaO2 (p= 0.001, 0.002, respectively) in patients compared to controls. The baseline characteristics, clinical and laboratory data of patients and controls are shown in **Table.1**.

**Table-1**: Baseline characteristics, clinical and laboratory data of patients and controls.

| Characteristics | Patients, n=40 | Controls, n=40 | P- value |
|---|---|---|---|
| Age in months | 40.6±8.6 | 20.5±16.1 | 0.3 |
| Sex (n): Males<br>        Females | 23(57.5%)<br>17(42.5%) | 28(70%)<br>12(30%) | 0.2 |
| Weight kg | 11.72± 4.89 | 12.83 ± 3.26 | 0.2 |
| Heart rate beats/m | 141.4± 18.4 | 102.6 ± 14.7 | 0.01 |
| Mean temperature (℃) | 38.8±1.5 | 36.8±0.5 | 0.02 |
| Respiratory rate | 55.5 ± 10.3 | 30.60 ± 5.65 | 0.01 |
| Clinical presentation<br>Lobar pneumonia<br>Bronchopneumonia<br>Interstitial pneumonia | 15(37.5%)<br>18(45%)<br>7(17.5%) | | |
| Hb gm/dl | 9.1±1.3 | 10.8±1.5 | 0.02 |
| TLC (x1000/dl) | 12.3±4.1 | 7.2±2.3 | 0.001 |
| Neutrophil count (x1000/dl) | 7.3±5.1 | 4.2±1.5 | 0.001 |
| CRP mg/dl | 33± 40.8 | 7± 0.5 | 0.001 |
| Mean O2 saturation | 84.9±14.3 | 97.7± 2.1 | 0.01 |
| Mean PaO2 mmHg | 63.8±19.8 | 94.9±2.6 | 0.001 |
| Mean PCO2 mmHg | 35.1±12.9 | 36.9±4.9 | 0.01 |
| CD64 expression on neutrophils (MFI) | 1140±109.7 | 327.3± 25.7 | 0.001 |

PaO2= partial pressure of arterial oxygen; PCO2=partial pressure of carbon dioxide.

## 3-2. Neutrophil CD64 expression

The mean value of MFI of nCD64 expression in patients with CAP was (1140±109.7) (RFU) which was significantly higher than healthy controls (327.3± 25.7) (p= 0.001) (**Table.1**). A trend towards greater nCD64 expression was observed in patients with clinical deterioration and ICU admission (group 2) MFI (1885.3± 870.2) **Table.2**. Significant positive correlations were found between CD64 and CRP, and total leucocytic count of patients. Significant negative correlations were present between CD64

and platelet count, age and weight of patients (**Table.3 and Figure.1**). ROC curve (**Figure.2**) showed area under curve (AUC) of 0.62, and sensitivity and specificity of 70% and 85%, respectively, for diagnosis of pneumonia.

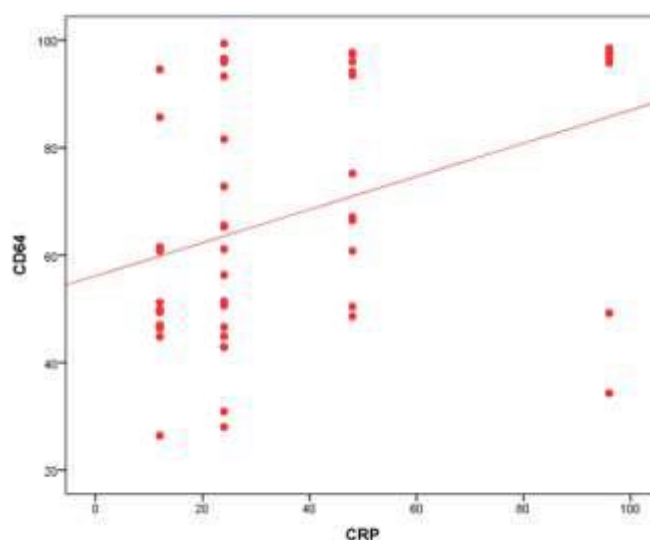**Table-2**: Level of CD64 in patients according to the severity of the disease.

| Parameter | Group 1, n=30 | Group 2, n= 10 | Group 3, n=40 | P-value Between Groups | | |
|---|---|---|---|---|---|---|
| | | | | 1 and 3 | 2 and 3 | 1 and 2 |
| CD64 (MFI) | 927.3±550.7 | 1885.3±870.2 | 327.3±25.7 | 0.001 | 0.000 | 0.001 |

MFI: mean fluorescence intensities; Group 1; patients without clinical deterioration; Group 2; patients in intensive care unit (ICU); Group 3; controls.

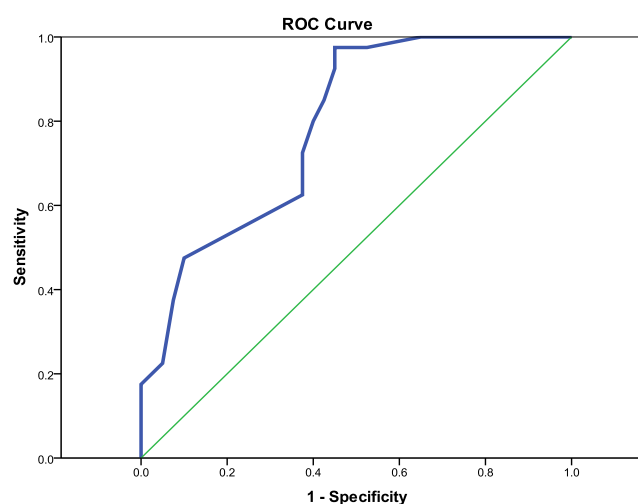**Table-3**: Correlation between neutrophil CD64 and other studied parameters.

| Parameters | CD64 (RFU) | |
|---|---|---|
| | Pearson correlation coefficient | P- value |
| Age, month | 0,9 | 0.001 |
| Weight, kg | 0.8 | 0.01 |
| Platelet (x1000/d)) | 0.7 | 0.008 |
| Total WBC (x1000/dl) | 0.76 | 0.01 |
| CRP (mg/l) | 0.76 | 0.001 |
| PRESS (Pediatric Respiratory Severity Score) (ranged 0 to 5). | 0.220 | 0002 |

WBC: white blood cells; CRP: C-reactive protein.



**Fig.1**: correlation between neutrophil CD64 and CRP.

CRP: C-reactive protein.

**Fig.2**: ROC curve analysis nCD64 level in CAP patients.
CAP: Community-acquired pneumonia. nCD64: Neutrophil CD64.

## 4- DISCUSSION

Community-acquired pneumonia is a vital feature as it is one of the most common causes of mortality among other infectious diseases in the developed countries and is globally the second biggest cause of death (14). In recent years, several biomarkers have been evaluated as diagnostic tools and for prognostic purposes in patients with infection and sepsis; an ideal biomarker should improve the initial diagnostic assessment of patients presenting with an infection and lead to institution of antibiotic therapy, with appropriate duration of therapy, and the identification of patients with the most severe conditions requiring intensive care (20). Neutrophil CD64 expression was studied as an early marker of bacterial infection and sepsis (21, 22). It has negligible expression in healthy subjects, with prompt upregulation under inflammatory cytokine stimulation (23). It is stable at room temperature for more than 30 hours, indicating favorable properties as a good marker. Moreover, neutrophil CD64 expression seems to be useful in distinguishing systemic infections from other inflammatory conditions (24, 25). To the best of our knowledge, studies that assess the role of nCD64 expression as a prognostic marker in children with CAP are scarce, so the aim of our study was to evaluate the diagnostic and prognostic value of nCD64 in children with pneumonia. The results of our study revealed that the level of CD64 is significantly elevated in patients with CAP when compared to controls. These findings are in agreement with Burgos et al. (26), and Christ-Crain and Müller (27). Gámez-Díaz et al. (28) in a study including septic patients recently admitted to the emergency department (ED), reported a sensitivity of 65.8% and a specificity of 64.6% for CD64 as predictive marker of infection defined by an experts' consensus diagnosis. In the population studied, 22% of patients were affected by community-acquired pneumonia and the median CD64 was MESF (molecules of equivalent soluble fluorochrome) units in this group. Also, a higher CD64 expression was found among patients who met criteria for infection during the 72-h period of observation but who were not considered infected during the initial evaluation. Compared with white blood cell count, neutrophil count, the CD64 expression was

found to be a good predictor of infection. Although Davis et al. (29), showed a weak correlation between CD64 and neutrophil count, this can be explained by the different specificity for detection of an acute inflammatory response or a different kinetic during the dynamic process of inflammatory response to infection. CRP, a globulin produced by the liver during any generalized inflammatory process, as a result of stimulation by IL-1 and IL-6, increases only after 12-24 h from the onset of infection. This limits its use in the initial evaluation of infection, however, serial measurements of CRP are useful in monitoring the progress of infection (30).

In the current study, CD64 expression had a significant correlation with CRP levels pointing to its usefulness as an additional marker of pneumonia; this is in agreement with Kantar et al. (31). Also, our results revealed a sensitivity and specificity of 100% each for CD64 expression in community acquired pneumonia, higher than those reported by Ng et al. (97% and 89% respectively) (32), Lino et al. (33), and Chan et al. (34); also our study included patients with severe infection. To the best of our knowledge, studies that assess the role of nCD64 expression as a prognostic marker in patients with CAP are scarce. In the present study, we observed that nCD64 expression in patients with CAP was increased in those individuals that required ICU admission or presented clinical deterioration after admission. This is in agreement with Chan et al.'s study (34).

## 4-1. Study Limitations

Some limitations of our study must be pointed out. Firstly, a limited number of patients could preclude finding associations between nCD64 expression and different outcomes. Secondly, this is a single center study and uses a non-standardized flow cytometry method, making its results less generalizable to other settings. Finally, nCD64 expression

was measured by trained laboratory personnel.

## 5- CONCLUSION

Based on the results, nCD64 expression was sensitive and specific for early diagnosis of community acquired pneumonia and patients with higher levels were associated with an increased risk of ICU admission and clinical deterioration after admission.

## 6- CONFLICT OF INTEREST: None.

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