The role of nCD64 in the diagnosis of neonatal sepsis in preterm newborns

Jan Halek[a], Martin Novak[b], Anna Medkova[a], Tomas Furst[c], Jarmila Juranova[b]

Background. Diagnosing neonatal sepsis is difficult, particularly in preterm newborns. A promising method appears to be evaluation of cell surface markers by flow cytometry.
Methods. This prospective study investigated 217 newborns suspected of having early- or late-onset neonatal sepsis. In all, flow cytometry was used to determine the proportion of CD64-positive neutrophils (nCD64). Based on the clinical course and laboratory test results, newborns were categorized as having proven, possible, clinical or no neonatal sepsis. Subsequently, associations between the categories and nCD64 values were analyzed.
Results. There were significant associations between nCD64 values and the development of sepsis in newborns with both early- or late-onset sepsis.
Conclusion. nCD64expression is significantly elevated in preterm newborn with early and late onset sepsis. The results show that nCD64 is a reliable marker for diagnosing neonatal sepsis.

Key words: CD64, neonatal sepsis, preterm newborn

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[a]Neonatal Ward and Department of Pediatrics, University Hospital Olomouc and Faculty of Medicine and Dentistry, Palacky University Olomouc, Czech Republic
[b]Department of Hemato-Oncology, University Hospital Olomouc and Faculty of Medicine and Dentistry, Palacky University Olomouc, Czech Republic
[c]Department of Mathematical Analysis and Mathematical Applications, Faculty of Science, Palacky University Olomouc, Czech Republic
Corresponding author: Jan Halek, e-mail: jan.halek@fnol.cz

BACKGROUND

Neonatal sepsis (NS) is a rather serious but relatively common health problem. The incidence is around 2.7%, being particularly high in the population of very-low-birth-weight (VLBW) infants[1,2]. The consequences of NS can be minimized by early initiation of antibiotic therapy. Due to high NS rates, vulnerability of the organism in the neonatal period and concerns about consequences (considerable mortality, association with other acute or chronic complications, etc.), antibiotic therapy is commonly started in clinical practice even though nonspecific clinical signs develop. This is in spite of the fact that antibiotic overuse is linked to major negative outcomes (development of necrotizing enterocolitis, emergence of resistant bacterial strains, impact on the neonatal microbiome, etc.) (ref.[3,4]). The reliable and early diagnosis of NS is therefore essential but, unfortunately, rather difficult. Traditionally, it relies on identifying the pathogen causing bacterial infection in the bloodstream and the presence of typical clinical signs. However, the sensitivity of blood culture is low, particularly in early-onset sepsis (EOS), mainly due to antibiotic therapy frequently administered to mothers in the perinatal period, varying bacterial counts in samples and technical difficulties such as collecting enough blood, particularly in VLBW neonates. Another drawback of classical bacteriological tests is their duration since it takes 48 to 72 h from collection to identify a sample as clearly negative[5]. In the neonatal period, the clinical signs of infection are nonspecific, once again, mainly in preterm newborns; they overlap with other immaturity complications (infant respiratory distress syndrome, circulatory instability, patent ductus arteriosus, impaired thermoregulation, etc.) (ref.[6,7]). Given the pitfalls of diagnosing NS, novel markers are searched for and used. Under ideal circumstances, these should fulfill the following criteria: Such markers should allow diagnosis in both the early and late stages of infection and timely response to adequate therapy. The sensitivity of an ideal marker should approach 100%, and so should its specificity. With regard to patients, the marker should be detected in small sample volumes; the tests should be rapid, simple and inexpensive. These criteria are not met by any of the currently used markers. The so-called classical hematological markers (leukocyte, neutrophil and platelet counts, I:T ratio, immature leukocyte count, various hematological indices, etc.) are commonly included in examination patterns but their diagnostic accuracy and specificity are limited. Probably the most widely used "biochemical" marker of NS, C-reactive protein (CRP), is one of the so-called late markers. Its sensitivity is mainly low in the early stages of infection; its reliability increases, particularly with serial measurements. In that case, its negativity practically rules out the presence of NS. It is not completely specific for NS (ref.[7-9]). Procalcitonin (PCT), an intermediate marker, is relatively specific, providing prognostic information as well; it decreases rapidly in response to effective therapy. However, its complex postnatal "physiological" dynamics makes its measurements difficult, particularly in EOS (ref.[10,11]). Among cytokines, interleukin 6 (and, possibly, interleukin 8) seems to be most commonly used in practice. This very early NS marker is pathophysiologically

involved in the initial phases of the organism's response to microorganism invasion. The drawback is its very fast dynamics as it rapidly decreases after the initial phases of the infectious process. Therefore, it should be used in combination with an intermediate or late marker (PCT, CRP) (ref.[4,7,8,12]). Studies have tested the use of numerous other potential markers such as proteins, cytokines, metabolomic and genomic markers. Additionally, molecular genetic methods such as polymerase chain reaction or sequencing are definitely promising as these may improve pathogen detection, particularly in EOS (ref.[13,14]).

Flow cytometry is a bioanalytical method which combines the principles of fluorescence microscopy and haematological analyser. It is based on measuring and subsequently analysing the physical characteristics of cells in the carrier fluid in interaction with light radiation. The basic quantities usually analysed are the size of the cells, the density of their inner content (granularity) and the intensity of fluorescently marked monoclonal antibodies for the detection of complementary antigens on the surface and inside the analysed cells. Given its high sensitivity, rapid laboratory response, wide range of analytical possibilities and also relatively low cost of an examination, flow cytometry became a "gold standard" in many branches of biometric analysis.

The organism's response to pathogenic strain invasion may be recognized early by detecting surface markers on cells involved in the immune response. Numerous markers on the surface of leukocytes and macrophages have been studied, one of them being CD64. Also known as Fc-gamma receptor 1, CD64 is a high-affinity receptor for monomeric IgG antibodies, thus involved in the process of phagocytosis of opsonized bacteria. Since the expression of the receptor on neutrophil surfaces increases approximately one hour after invasion, it may be a relative early marker of infectious complications. Reports on the use of CD64 expression on neutrophils (nCD64) in diagnosing NS are somewhat contradictory, yielding inconsistent results[13]. The presented pilot study aimed to verify whether one of possible methods for nCD64 measurements may be used for diagnosing NS in preterm newborns.

MATERIALS AND METHODS

Patients

The study analyzed blood samples collected from preterm neonates (born before gestational week 34) cared for in the University Hospital Olomouc Neonatal Ward between March 2016 and June 2017. There were two groups of patients: (1) According to the standard ward routine, blood samples were collected 12 to 14 h postnatally for blood cell counts, biochemical analysis and EOS diagnosis. (2) Blood samples were collected for repeated laboratory tests or in case of suspected late-onset sepsis (LOS) at any time but no sooner than 72 h postnatally.

Methods

The analysis utilized samples collected to determine the numbers of blood elements and perform differential blood count. In all samples, assessment and quantification of given surface cell markers were carried out by means of flow cytometry. Analysis of the acquired samples was carried out by means of multicolour flow cytometry with an antibody panel against CD3, CD4, CD8, CD16+CD56+, CD14, CD15, CD163, CD19, CD138, CD45RO, CD64 and the appropriate isotope controls (EXBIO, eBioscience, BD Biosciences) on a cytometer FACSCanto II (Becton Dickinson). The samples were processed by a lyse-no-wash method, a volume of 50 μL of peripheral blood was incubated with the antibodies for 30 minutes in the dark at the temperature of 5 °C, and subsequently, erythrocytes were lysed by adding lysing solution BD FACS lyse (Becton Dickinson) for 30 minutes in the dark at the temperature of 25 °C.

The proportion of the target subpopulation CD64 on CD15-positive granulocytes was determined by means of the analytical software FACS Diva ver. 8.0 1 (BD Biosciences), while at least 50 000 events in the granulocyte gate were collected for the data analysis. CD64 positivity was assessed by median fluorescence intensity, with T-lymphocytes as the population of reference.

The other diagnostic and therapeutic procedures were carried out in accordance with the previously established ward standards; these included common biochemical marker measurements. Prior to therapy, blood samples were collected for microbiology tests.

Subsequently, the disease status and course was assessed and, based on the clinical course and laboratory test results, newborns were categorized as follows:

A: Possible (clinical) sepsis (infection without proof of pathogen): All of the following criteria must be met:

1. Treating physician institutes appropriate antimicrobial therapy for bloodstream infection for at least 5 days
2. NO pathogens detected in blood culture or blood cultures not performed
3. NO apparent infection at another site AND two of the following criteria must be met:
   a. Fever or temperature instability (frequent incubator adjustment) or hypotermia
   b. Tachycardia or new/more frequent bradycardia
   c. Recapillarization time >2 s
   d. New or more frequent apnea (>20 s)
   e. Other signs of bloodstream infection: skin color, laboratory evidence (CRP, interleukin), increased O2 requirement (intubation), unstable condition, apathy

B: Proven sepsis (Laboratory-confirmed bloodstream infection with proof of pathogen) Pathogen isolated in blood culture or cerebrospinal fluid (pathogen not related to infections at other sites) AND two of the following criteria

a. Fever (>38 °C) or temperature instability (frequent incubator adjustment) or hypothermia
b. Tachycardia (> 200/min) or new/ more frequent bradycardia
c. Recapillarization time >2 s
d. New or more frequent apnea (>20 s)

e. Other sign of bloodstream infection: skin color (only when recapillarization time is not used); laboratory findings (CRP, interleukin), increased oxygen requirement (intubation), unstable condition, apathy

C: No sepsis (negative clinical and laboratory signs, negative blood culture) (ref.[15,16]).

Statistical analysis

Differences in continuous variables between groups (e.g. difference in nCD64 expression between patients with and without sepsis) were determined using boxplots and the Kruskal–Wallis test. Standard ROC curves were created as follows: for all possible cutoff values of the tested predictor, 1–specificity (*x* axis) was plotted against sensitivity (*y* axis). Given the small number of patients, data visualization was generally preferred to hypothesis testing. 0.05 was the significance level. All analyses were conducted using MATLAB 2013b and Statistics Toolbox (The MathWorks, Inc., Natick, Massachusetts, United States).

Ethics

No additional sample collection was necessary as the analyses were performed using material obtained by blood sampling in accordance with the standard ward routine. Complementary examinations were consistent with informed parental consent.

RESULTS

Group 1 comprised a total of 174 patients with a mean birth weight of 1613 g (410-2450) and a mean gestational age at birth of 13 weeks (24-33), of whom 23 had proven (n=1) or possible (n=22) sepsis; the others had no infectious complications. In all 23 patients with proven or possible sepsis, antibiotic therapy was initiated according to the ward standards. Statistical analysis clearly showed

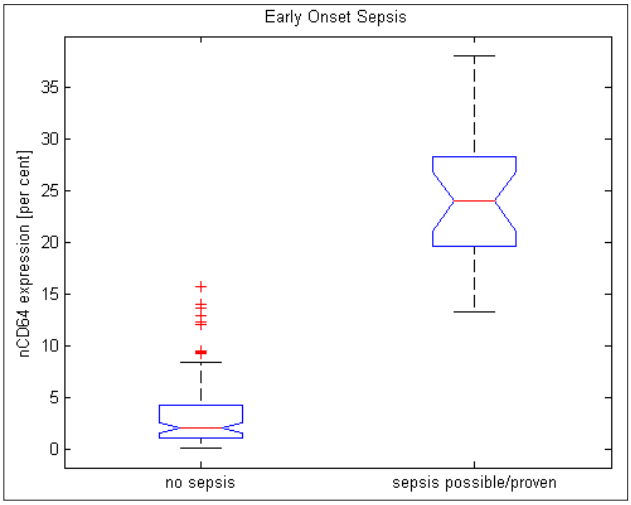

Fig. 1. nCD64 values: comparison of EOS patients with and without sepsis. The difference in nCD64 expression is statistically significant at a significance level of 10^{-13}.

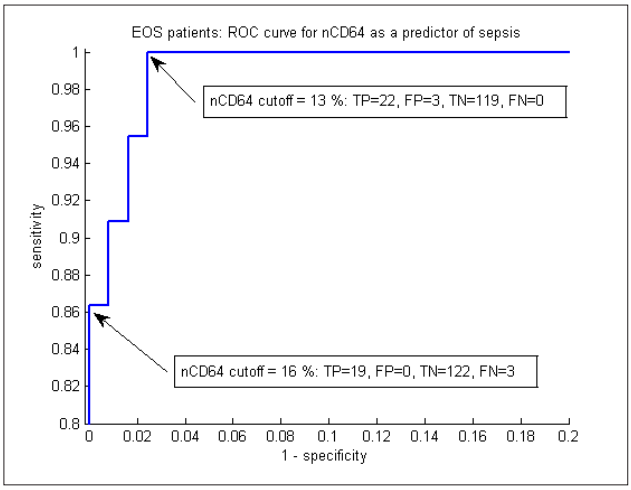

Fig. 2. A portion of the ROC curve: comparison of sensitivity and specificity for various nCD64 cutoffs; true positive (TP), false positive (FP), true negative (TN) and false negative (FN) cases.

a significant association between nCD64 values and the presence of sepsis, either proven or possible (Fig. 1).

A portion of the ROC curve (Fig. 2) indicates, once again, an excellent predictive value of nCD64 expression for NS (proven or possible). The ideal cutoff appears to be 13% (sensitivity and specificity, negative and positive predictive values).

Group 2 (LOS) comprised 43 patients with a mean birth weight of 1282 g (410-2200) and a mean gestational age at birth of 31 weeks (24-33), of whom 4 had proven and 4 had possible sepsis; the other 35 patients had no sepsis. Once again, nCD64 values were clearly associated with sepsis (proven or possible, $P<0.0001$); also significant was the difference in nCD64 values between the two groups of patients with sepsis ($P=0.0001$, see Fig. 3). Due to the small number of patients, the ROC curve was not plotted and assessed. In these patients, another analysis compared nCD64 association with CRP and leukocyte count performed at the same time. The association was closer for nCD64 than for CRP or leukocyte count (Fig. 4 and 5).

DISCUSSION

There is increasing evidence in the literature to support the use of nCD64 for diagnosing sepsis in general as well as neonatal sepsis, in both full-term and preterm newborns[15,17-22]. The data obtained so far show that this marker has a relatively early increase; the advantage is that its values are stable in the early postnatal period[23]. Study results are inconsistent, possibly due to different methods used to measure nCD64 (ref.[24-27]) or, especially, due to low homogeneity of study samples. A great proportion of the published studies fail to properly consider different patient groups (preterm vs full-term newborns), disease pathophysiology (in case of EOS, the disease has different dynamics depending on whether infection develops prenatally or is due to postnatal microbial invasion; LOS)

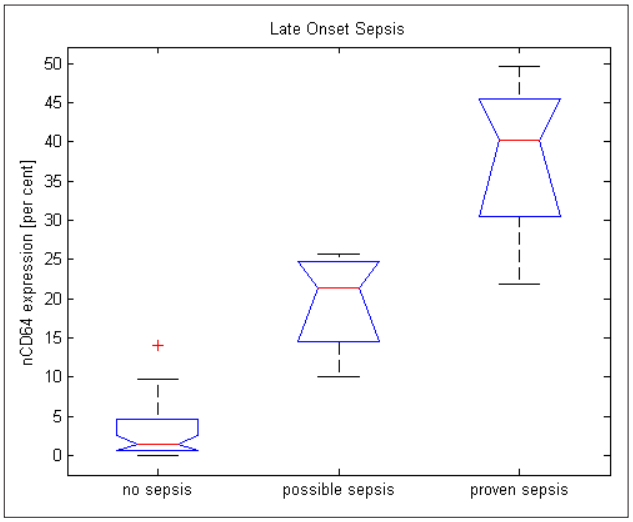

Fig. 3. Comparing nCD64 expression values between LOS categories; the differences are significant at a level of P=0.0001.

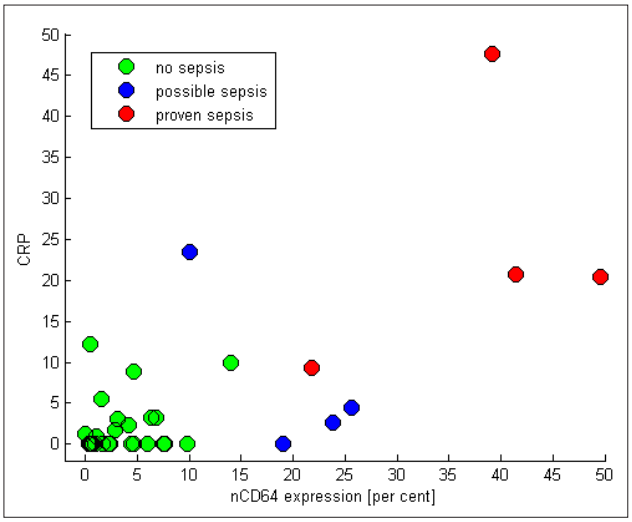

Fig. 4. Comparing markers (CRP [mg/L] and nCD64) and their yields in the diagnosis of LOS. Expression of nCD64 (vertical distribution) is clearly better at stratifying patients than CRP (horizontal distribution).

or time of sample collection, ignoring the dynamics of increase in markers of infection.

So far, two meta-analyses have been published. Surprisingly, even those do not provide consistent conclusions. One considers nCD64 a marker of little benefit[28] while the other reports its excellent diagnostic accuracy[29]. Comparison of the yields of nCD64 and CRP in the diagnosis of LOS shows a closer association in case of nCD64, reflecting the fact the rise of CRP after bacterial invasion is slower in the neonatal population, making it the so-called late marker of infection[30].

The present study's results show a very close and significant association between nCD64 and NS. For several reasons, however, the findings cannot be generalized. First, our method is different from those commonly used to measure nCD64 (ref.[24-26]) as it is based on the proportion of CD64-positive neutrophils which is not an index most frequently compared with the standard. Second, the study sample was small and, moreover, the NS rate was relatively low. Therefore, more accurate criteria and larger samples will be needed in further studies.

Based on our findings, flow cytometry proves to be a useful tool for early and sensitive detection of neonatal sepsis. Currently, there are however only a few facilities able to carry out comprehensive multicolour examination by flow cytometry, especially due to difficult method standardization and data interpretation, which require long-term training of laboratory staff. Due to the same reasons, examinations are available almost only during the usual working hours, which reduces the potential to rapidly detect sepsis markers in the time window of 1-12 h after the invasion of pathogens into the blood flow. Nevertheless, rapid development of technology in the field of flow cytometry, especially in analytical software and algorithm assessment, bears the promise of fast progress and therefore better availability of even comprehensive assessments also in this field.

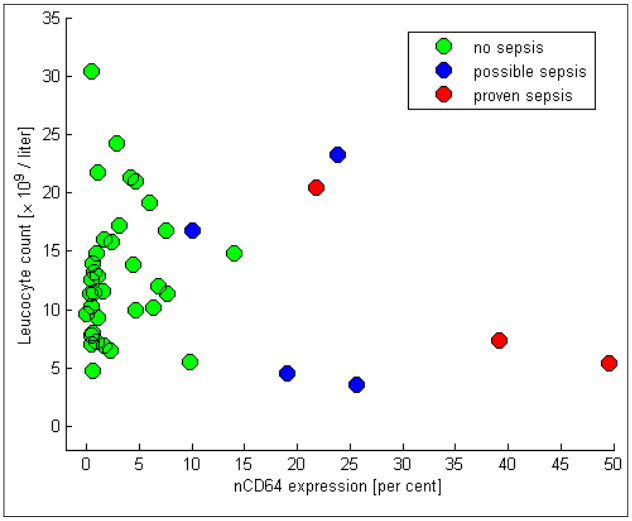

Fig. 5. Comparing markers (leukocyte count and nCD64) and their yields in the diagnosis of LOS. Expression of nCD64 (vertical distribution) is clearly better at stratifying patients than leukocyte count (horizontal distribution).

CONCLUSION

nCD64 expression is closely and significantly associated with neonatal sepsis in preterm newborn. The present study's results show nCD64 to be a useful tool for early and sensitive detection of neonatal sepsis in the specific population of preterm newborn. Before it is introduced into routine clinical practice, however, further investigation is needed – with more accurate criteria and larger samples.

Abbreviations: NS, Neonatal sepsis; VLBW, Very low birth weight; EOS, Early-onset sepsis; CRP, C-reactive protein; PCT, Procalcitonin; LOS, Late-onset sepsis; TP, True positive; FP, False positive; TN, True negative; FN, False negative.

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