Sys Rev Pharm 2020;11(9):1077-1081
A multifaceted review journal in the field of pharmacy

nCD64, mHLA-DR: Sensitive Diagnostic Markers of Infection in Term Infants Receiving Antibiotic Treatment

Nguyen Thi Ngoc Tu*, Le Thanh Hai*, Truong Thi Mai Hong*, Pham Thu Hien*, Le Thi Ha*, Doan Thi Mai Thanh*

* Vietnam National Children Hospital
Corresponding author: Nguyen Thi Ngoc Tu
Email: nguyenthingoctu.nhp@gmail.com

ABSTRACT

The diagnosis of neonatal sepsis is difficult because of its nonspecific clinical pictures. Monocytic human leukocyte antigen–DR (mHLA-DR), neutrophil surface CD64 (nCD64) expressions and their ratio (SI), have been used to identify newborns with sepsis. However, there is a lack of studies investigating the expression of these markers in neonatal patients receiving antibiotic treatment. The main aims of the study are: to determine the sensitivity, specificity value of nCD64, mHLA-DR and SI using the QuantiBRITE Anti HLA-DR/Anti-Monocyte, a Becton Dickinson novel reagent that standardizes flow cytometry values for diagnosing infection in term newborns receiving antibiotics; to define the optimal cutoff value of these markers using the receiver operating characteristics (ROC) curve. We determined mHLA-DR and nCD64 expressions in 44 term infants with sepsis receiving antibiotic treatment admitted to neonatal intensive care unit at National Children Hospital, Hanoi, Vietnam, between December 2019 and March 2020. mHLA-DR, nCD64 expressions were quantified on the admission day and the SI (mHLA-DR*100/nCD64) was calculated. We also measured mHLA-DR, nCD64 levels and SI in 17 non-sepsis control patients. Mean nCD64 expressions was significantly lower in septic patients than in controls (p = 0.004). By contract, mHLA-DR expression was notably higher in sepsis group. Sensitivity and specificity to detect sepsis using nCD64 was 73.5% and 88.2%, respectively, while for SI it was 91.2% and 100%, respectively. Specificity was highest for SI>17 (100%). Positive likelihood ratios were highest for nCD64 > 3427.4 ABC (88.2%). We found out that nCD64, SI were beneficial for diagnosis of sepsis in neonatal patients receiving antibiotic whereas mHLA-DR was a marker of low diagnostic value. If further validated, the use of nCD64 and SI as infection markers should allow indication of antibiotic treatment.

Keywords: Monocytic human leukocyte antigen–DR; neutrophil surface CD64; sepsis; infants; antibiotic treatment

Correspondence:
Nguyen Thi Ngoc Tu
Vietnam National Children Hospital
Email: nguyenthingoctu.nhp@gmail.com

INTRODUCTION

Neonatal sepsis remains one of serious problems leading to morbidity and mortality among children under 5 years in low and middle-income countries as Vietnam [1].

The diagnosis of neonatal sepsis is difficul because of its nonspecific clinical pictures, particularly in the patients receiving antibiotics because medicine changes the investigated results. Blood culture considered gold standard of bacterial infection with the low specificity of 20-25%, could be even lower due to antibiotic effecting [2].

Flow cytometry-based monitoring with biomarkers has provided tools to assist the clinical decision making in sepsis with nCD64 and mHLA-DR. While nCD64, the Fc-gamma receptor 1 on neutrophils, has been shown significant increased expression in response to sepsis [3,4], a decrease in mHLA-DR, monocytic human leukocyte antigen–DR, has been associated with poor outcome in newborn infections without antibiotic treatment [5]. However, in the patients receiving antibiotics, there is lack of such evidence.

We hypothesized that markers of sepsis monitored by flow cytometry including nCD64 and HLA-DR have good tools in diagnosis of infection in term neonates receiving antibiotics.

This prospective study aimed:

- To determine the sensitivity, specificity value of nCD64, mHLA-DR and their ratio SI for diagnosing infection in receiving antibiotic newborns.
- To define the optimal cutoff value of these markers using the receiver operating characteristics (ROC) curve.

MATERIALS AND METHODS

A prospective study was conducted on 51 neonates who were admitted Neonatal Intensive Care Unit (NICU) of Vietnam National Children Hospital (VNCH) from December 2019 to April 2020.

Patients

Neonates (<28 days) of either sex having a suspicion of sepsis receiving antibiotic from local hospital to VNCH were recruited irrespective of their birth weight, day of life and gestational age who presented at least two clinical symptoms and at least two laboratory signs as under (met the EMA 2010 criteria):

The clinical criteria included:

Modified body temperature:

- Core temperature greater than 38,5 °C or less than 36 °C AND/OR temperature instability
- Cardiovascular Instability:

Bradycardia (mean HR less than the 10th percentile for age in the absence of external vagal stimulus, beta-blockers or congenital heart disease OR otherwise unexplained persistent depression over a 0.5 h time period) OR, tachycardia (mean HR greater than 2 SD above normal for age in the absence of external stimulus, chronic drugs and painful stimuli OR otherwise unexplained persistent elevation over a 0,5 h to 4 h time period) AND/OR rhythm instability, reduced urinary output (less than 1 mL/kg/h), hypotension (mean arterial pressure less than the 5th percentile for age), mottled skin, impaired peripheral perfusion

- Skin and subcutaneous lesions: petechial rash, sclerema
- Laboratory signs: White blood cell (WBC) count > 20000 ox 10^9cells/L or under 4000 x

10^9cells/L, immature to total neutrophil ratio (I/T) > 0.2 , platelet count < 100 x 10^9cells/L, crp > 15mg/L, glucose intolerance confirmed at least 2 times hyperglycaemia (blood glucose >180mg/dL or 10 mMol/L) or hypoglycaemia (glycaemia< 45 mg/dL or 2.5mMol/L) when receiving age specific normal range glucose amount, base excess (BE) <-10 mEq/L or serum lactate >mmol/L

Exclusion criteria: major congenital anomaly, inborn errors of metabolism, neonates who received blood transfusion.

Time Points for Sampling of Blood

On the admission day to NCH, blood samples were processed including blood cultures, hematologic laboratory investigations (differential white blood cell count, platelet count, absolute neutrophil count, immature/total (I/T), C-reactive protein, and serum glucose concentration. In addition, examination of CSF for protein, glucose, arterial blood gas, total, urine fluid etc., were measured if necessary. Chest x-rays and other investigations were performed whenever patients presented with signs suggested.

Classification of Infection Episodes

Three categories of "infection" episodes were defined prospectively in this study.

Group 1 (define infected group).

The infected group consisted of infants who had microbiologic-confirmed bacterial infections (exclude skin commensal) including blood culture, cultures of cerebrospinal fluid (CSF), urine, endotracheal tube, central catheter tips abdomen, pleural effusion, pus. Other infections as necrotizing enterocolitis (NEC - stage II or above in Bell's classification with or without positive blood culture), pneumonia, abscess of skin, urine infection was also included in this group with or without positive culture results.

Group 2 (suspected infected group).

Cases that failed to meet the criteria for the "define infected" group were considered as suspect infected group.

Group 3 (noninfection group).

Infants admitted NCH having no suspicion of sepsis, but chief complain of the other diseases as tumor of mediastinum, trauma of accident, re-examine in follow up program of HIE after discharge, screening for G6PD deficient …

Blood culture was performed Vitek 2 compact for patients in group 1 and 2. Wherever clinically indicated, cultures of cerebrospinal fluid (CSF), urine, endotracheal tube, central catheter tips and pus were performed. They were considered positive when bacteria isolated were not skin commensals. If the culture was a skin commensal, a repeat culture was performed.

CRP was measured in Adiva 1800, Siemens, Japan.

WBC count, platelet count and I/T ratio were measured in Advia 2120i, Siemens, Ireland

FCM Analysis to monitor nCD64, mHLA-DR

Samples were stored in EDTA tubes at 4°C and analyzed within 2 to 8 hours of collection, using the QuantiBRITE Anti HLA-DR/Anti- Monocyte

Statistical Analysis

These results were performed by SPSS 20.0 statistical software (SPSS Inc, IL). The level of significance considered was 0.05. Sensitivity, specificity was used to compare in both the individual biomarkers and the combine of two or more ones. Receiver operating characteristic (ROC) curve was used to assess the accuracy and the cutoff point of each maker (nCD64, mHLA-DR, SI) or combination of them in prediction of neonatal sepsis.

According to EMA 2010 criteria, we chose cut-off of CRP, WBC and PLT were 15mg/l, 20000/mm^3 and 100000/mm^3, respectively.

RESULTS

From 12/2019-4/2020, a total of 51 neonates were studied, of which 22 and 12 were define sepsis (group 1) and suspected sepsis (group 2) respectively. 17 of whom belonged to non-sepsis (group 3). The clinical characteristics of the three study groups are summarized in Table 1.

Table 1. Clinical characteristics of study population

Parameter	Define sepsis (Group 1)	Suspected sepsis (Group 2)	Nonsepsis (Group 3)	P
Number of infants (n)	22	12	17	>0.05
Post-natal age (day)	6.2 (1-25)	4.3 (1-20)	18 (1-25)	>0.05
Gender male:female (n:n)	10:12	2:10	8:9	>0.05
Gestational age (wk)	38.4	38.6 (37-41)	38,1 (37-41)	>0.05
Birth weight (gram)	2726 (1900-3800)	2804.17 (2000-3700)	3011.76 (2600-3600)	>0.05

No significant differences in age, gender, post-natal age and birth weight were observed between septic patients and controls (p>0.05)

Medium antimicrobial therapy duration before admission in group (1) and (2) was 2.3 days, from 1 day to 12 days.

Table 2: Levels of biological markers of sepsis evaluation on admission day

Parameter (X ± SD)	Define sepsis (Group 1)	Suspected sepsis (Group 2)	Nonsepsis (Group 3)	P
nCD64 (ABC)	5648.22 (978- 14991)	4635.83 (593-15044)	2135.70 (272-7330)	0.04
mHLA-DR (*ABC)	11306.68	13823.66	42708.29	0.00

	(2131-24598)	(1330-56363)	(10863-93049)	
SI	85.81 (8.6-299)	115.97 (1.5-692.1)	5.62 (1.5-13.9)	0.01
CRP (mg/dl)	60.64 (1-238)	16.47 (0.38-114.34)	1.04 (0.02-4.13)	0.01
WBC (1000 cells/mm3)	19.28 (5.4-34)	15.01 (5.74-53.20)	14.01 (6.69-28.13)	0.32
PLT (1000 cells/mm3)	217.04 (4-599)	221.08 (16-429)	318.41 (154-537)	0.09

ABC: Antigen binding cell

Expression of nCD64 was significantly upregulated while mHLA-DR was significantly downregulated in the infected groups (Group 1 and 2) as compared to the non-infected group (Table 2). The Sepsis Index (nCD64 x 100/mHLA-DR) was significantly higher in infected than non-infected neonates (Table 2).

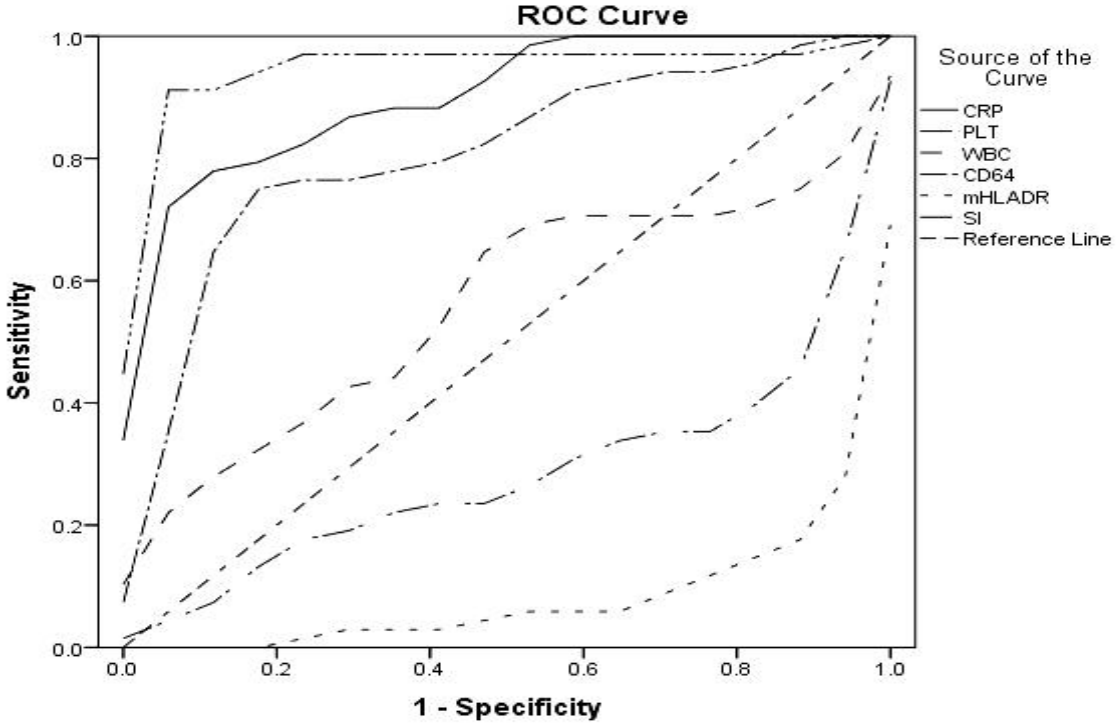

Figure 1. ROC curves of the biomarkers on admission day. WBC: white blood cell count; PLT: plalete count

The highest AUC was observed using SI followed by CRP then nCD64. The SI had AUC of 0.96, which was significantly higher than that of white blood cell count, plalete count ($p<0.0001$)

Table 3. Comparison of sensitivity, specificity of markers using cutoff values

Parameter	N	Sensitivity (%)	Specificity (%)
SI> 17	31	91.2	100
nCD64 >3427(ABC)	27	73.5	88.2
mHLA-DR ≤ 11189(ABC)	29	38.2	29.6
CRP>15mg/l	22	69.4	100
PLT<100(1000 cells/mm3)	37	61.8	94.1
WBC>20(1000 cells/mm3)	15	32.4	25.3
SI >17 and CRP ≥ 15mg/l	10	98	100

The ROC graph with the maximum area under the curve was chosen, and the optimal cutoff value for individual markers was then determined on the graph by minimizing the number of misclassified episodes for nCD64, mHLA-DR and SI (nCD64, 3427ABC; mHLA-DR, 11189ABC; SI,17). For WBC, PLT and CRP, we chose cut-off value according to EMA2010 criteria (WBC > 20000 cells/mm^3; PLT < 100000 cells/mm^3; CRP > 15mg/l). The assessment of individual markers indicated that CD64 has the highest sensitivity and specificity of 91.2% and 100%, respectively. Both CRP and PLT had the high specificity (100% and 94.1% respectively) contrary to low sensitivity (69.4% and 61.8%, respectively). In addition, the combination of SI and CRP provided the best values with sensitivity and specificity reaching 98% and 100%, respectively.

DISCUSSION

Diagnosis of neonatal sepsis is difficult owing to the non-specific symptoms [7] specifically in the patients receiving antibiotics. In this study, we investigated the diagnostic values of biomarkers individually and in combination by Flow cymetry in term infants with sepsis. Whereas none of CBC index cut-offs were associated with infection, we observed high sensitivity and specificity of SI (91.2% and 100%, respectively). Moreover, the use of SI in combination with other diagnostic marker such as CRP would be a "desired" test of its sensitivity and specificity exceeding 98% and 100%, respectively.

In general, our results are similar to those of previously published studies evaluating the nCD64, mHLA-DR and SI [5] and hematologic markers [8].

CRP is an acute phase protein synthesized in the liver in response to inflammatory cytokines. It takes 6 to 12 hours, even up to 24 hours for CRP to rise following onset of infection [9]. However, CRP may be elevated in some noninfectious conditions (prolonged rupture of membranes, maternal fever during labor, fetal distress, perinatal asphyxia, shock, intraventricular hemorrhage, pneumothorax, and meconium aspiration pneumonitis. Effat Hisamuddin reported the good correlation of patterns of CRP-ratio between before and after antibiotics treatment with the individual clinical course, patient outcome and also with the adequacy of antibiotic therapy [10]. CRP should be a crucial tool in the early identification of patients who had initial inadequate antibiotic therapy than an individual marker to identify sepsis. In this study, our patients in sepsis groups were transferred to our hospital owing to non-responding to treatment in local hospital. Therefore, we found the level of CRP was significant higher in sepsis groups with UAC of 91.2%. Using cut-off point of 15mg/l (EMA 2010), the specivity of CRP exceeded of 100% despite of the low sensitivity of 69.4%. Our results are the same with Effat Hisamuddin and Hofer [10, 11].

Bacteria or bacterial products may cause endothelial damage leading to platelet adhesion and aggregation or may bind directly to platelets leading to aggregation and accelerated clearance from circulation [12, 13]. Thrombocytopenia (platelet count < 100,000/mm^3) may be a presenting sign of neonatal sepsis and can last as long as 3 weeks; 10%-60% of infants with sepsis have thrombocytopenia [14]. However, thrombocytopenia is an insensitive and nonspecific finding. Our results showed no significant difference of plalete count between sepsis and non-sepsis group. Cut-off point of 100,000/mm^3 (EMA 2010) showed high specitivity of 94.1% but low sensitivity of 61.8% only. In the same way, Christoph P. Hornik reported the lowest positive likelihood ratios for plalete blood cell counts of < 50000/mm^3 [8]

Although white blood cell (WBC) counts and ratios are more sensitive for determining sepsis than platelet counts are, they remain very nonspecific value. Infants who related to the stress of delivery or to any of several other factors without infection may also demonstrate low WBC count (< 5000/mm^3) or elevated WBC count (>20,000/ mm^3). Tantiyavarong P. showed the constant trend of reduction of dialysate WBC over time of antibiotic treatment not depending on the outcomes[15]. We found no difference of WBC count of all groups in this study. Correspondingly, the immature-to-total (I/T) ratio which was considered as a good marker with sensitivity of 60-90% [16], remained <0.2 in both sepsis and non-sepsis group. Because of the absence of neutropenia in our study, the cut-off of WBC count of 20.000/mm^3 with its relatively low sensitivities (32.4%) and specificities (25.3%) made it unreliable to be used as an infection marker for identifying infection in patients receiving antibiotics. In like manner, Christoph P. Hornik investigated the highest cut-off for WBC count was 50000/mm^3 [8].

There is a markedly increase in CD64 expression on the surface of neutrophils in response to bacterial infection in neonates. Upregulation of CD64 on neutrophils (nCD64) is thought to be a very early step of host's immune response to bacterial infection, increasing approximately one hour after invasion and nCD64 expression is stable for more than 24 hours [17]. However, nCD64 expression shows rapid reduction after adequate antibiotic [18]. In this study, we found the expression of nCD64 was significantly increased in neonates with sepsis.

This figure was similar to those of previously published, larger studies evaluating the nCD64 in neonatal sepsis [5,19]. At the cut-off of 3427(ABC), we identified the sensitivity of 73.5% and specitivity of 88.2% little higher in Richeek Pradhan's research using cut-off of 92 MFI [5].

Although mHLA-DR downregulated markerbly in sepsis group to compare with non-sepsis group, its sensitivity and spectity was 25.39% và 83.33%, respectively in our study. Our results conflict to Ng et al with no significant difference in mHLA-DR expression between infected and non-infected or control groups [20] but similar to Richeek Pradhan's [5]. mHLA-DR showed as the marker of prognosis better than diagnosis [5, 21]

"Sepsis Index (SI)", the combination of pro-inflammatory markers like nCD64 and the anti-inflammatory response like mHLA-DR demonstrates physiological changes in sepsis. In this study, SI is a highly sensitive marker for the diagnosis of sepsis in term neonates receiving antibiotics. The level SI was independent of antibiotic therapy before with the sensitive of 91.2% and specitivity of 100%. Those result was higher to compare the two previous research [7]. Moreover, the combination of SI and CRP reached to sensitivity of 98% and specivity of 100%.

The strengths of our study include the quantitative flow cytometric analysis of leukocyte cell-surface antigens for diagnosis of sepsis in term infants receiving antibiotics. In addition, we report results on previously defined markers cut-offs commonly which are useful in clinical practice. This study was limited by the small sample with the patients in each group under 30.

CONCLUSION

In summary, we found that high SI and high nCD64 had an association with increased odds of infection in term infants receiving antibiotics. In the future, those indices should be further investigated and referred as a prospective routine biomarker in diagnosis of neonatal sepsis of indication of discontinuing antibiotic before identified bacterial culture results.

CONFLICTS OF INTEREST

The authors declare no conflict of interest

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