Original Article

Ratio of Neutrophilic CD64 and Monocytic HLA-DR: A Novel Parameter in Diagnosis and Prognostication of Neonatal Sepsis

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Objective: Approaches to monitoring of sepsis have traditionally relied upon the pro-inflammatory component of the sepsis response. This study evaluated the diagnostic and prognostic potential of the ratio of neutrophilic CD64 (nCD64) and monocytic HLA-DR (mHLA-DR) median fluorescence index in monitoring of neonatal sepsis.

Methods: Blood from 100 neonates suspected of sepsis and 29 healthy controls was collected on clinical suspicion of sepsis, and the expression of nCD64, mHLA-DR was evaluated by Flow Cytometry; thereby, a derived parameter "Sepsis index," SI = nCD64/mHLA-DR × 100 was estimated.

Results: At day 1, sensitivity and specificity to detect sepsis using nCD64 was 73.01% and 89.18%, respectively, while for SI it was 73.01% and 72.22%, respectively. On Kaplan-Meier analysis, neonates with SI > cut-off showed a higher 30 day-mortality than those with low SI ($P = 0.096$). On multivariate analysis, the factor associated with mortality in our cohort was Apgar score ≤3, while SI showed a trend toward significance.

Conclusions: At day1, nCD64 is useful for the diagnosis of neonatal sepsis whereas mHLA-DR is beneficial for monitoring patients at a later time point. The SI is a marker of moderate diagnostic sensitivity and supplements the current arsenal of laboratory investigations to detect neonatal sepsis. As a marker of prognosis, a high SI shows a trend towards greater mortality. © 2015 Clinical Cytometry Society

Key terms: neonatal sepsis; cd64; HLA-DR; sepsis index

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INTRODUCTION

Sepsis constitutes one of the leading causes of mortality and morbidity in the neonatal period worldwide (1–3). When left untreated, neonatal sepsis can potentially turn fulminant, making early diagnosis and intervention an essential component of the therapeutic strategy (4). Blood culture remains the gold standard for diagnosis of neonatal sepsis, but reports generally require 48–72 h in most settings. In addition, it has a

Additional Supporting Information may be found in the online version of this article.

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low sensitivity in detecting sepsis (5), thus limiting its application as a decision making tool for initiation of antimicrobial therapy. Accordingly, considerable attention has been focused on development of criteria for detection of infections in neonates to guide commencement of empirical therapy (4). On the other hand, since deterioration of health can be sudden and rapid, early prognostication of neonates is of crucial importance (6).

Flow cytometry based monitoring with biomarkers for sepsis has provided tools to assist the clinical decision making in sepsis (7) with CD64, the Fc-gamma receptor1 on neutrophils, emerging as a potential marker in the diagnosis of sepsis, its increased expression representing a pro-inflammatory response to infection (7–10). It has been shown to be effective in monitoring sepsis in critically ill adults with infection (11) and in serial monitoring of neonates (12). In adults, a raised CD64 on neutrophils has been associated with poor prognosis (11), while in neonates there is lack of such evidence (13). On the other hand, decreased expression of HLA-DR molecules on circulating monocytes has been associated with an anti-inflammatory immune response, referred to as "immunoparalysis" in sepsis (14,15). While the diagnostic significance of HLA-DR is limited (16), a decrease in HLA-DR on monocytes has been associated with deaths in adults (17) and newborn infections (18,19).

As progression and eventual mortality in sepsis is a trade-off between pro and anti-inflammatory responses, we hypothesized an ideal parameter to guide the management of cases should include monitoring of pro- and anti-inflammatory markers of sepsis such as CD64 and HLA-DR, respectively, i.e., ratio of nCD64 and mHLA-DR. The current study evaluated this parameter by flow cytometry to determine its diagnostic efficacy in sepsis in a neonatal intensive care unit (NICU) and its applicability in predicting prognosis in terms of mortality.

MATERIALS AND METHODS

Study Population

This prospective observational study was conducted from March 2012 to March 2013 in the NICU of Institute of Post Graduate Medical Education & Research (IPGME&R), Kolkata. Neonates (<28 days) of either sex having a suspicion of sepsis were recruited irrespective of their birth weight, day of life and gestational age. The clinical criteria included modified body temperature, core temperature >38.5 °C or <36 °C and/or temperature instability, cardiovascular instability (bradycardia, mean heart rate <10th percentile for the age in absence of external stimulus or congenital heart or otherwise unexplained persistent depression over 30 minutes or tachycardia, mean heart rate >2 SD above normal for age in the absence of external stimulus, drugs and pain or otherwise unexplained persistent elevation over a 30 minutes to 4 h and/or rhythm instability), reduced urinary output (<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), respiratory instability (apnea episodes or tachypnea episodes, mean respiratory rate (RR) over 2 SD above normal for age or increased oxygen requirements or requirement for ventilation support), gastrointestinal (feeding intolerance, poor sucking, abdominal distention) along with non-specific irritability, lethargy and hypotonia. When two or more of these criteria were positive, as per European Medical Agency (EMA) guidelines (20), blood was collected for culture and antimicrobials subsequently administered; cases receiving G-CSF therapy were excluded. Healthy controls comprised neonates having no suspicion of sepsis and were being routinely screened for congenital hemoglobinopathies. The study was approved by the Institutional Ethics Committee of IPGME&R, Kolkata; informed consents were obtained from the legally acceptable representatives.

Microbial Culture

On clinical suspicion of sepsis, blood culture was performed (BACTEC 9050, Becton Dickinson, MD). 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. For study classification, the false positive cases were considered as culture negative; if the culture report stated it was a skin commensal, yet the neonate appeared clinically septic, a repeat culture was performed.

Time Points for Sampling of Blood

Blood samples for flow cytometric (FCM) analysis were obtained on initial suspicion of sepsis, wherein blood was collected for culture and laboratory screening. Blood was also analysed for C-reactive protein (CRP), total leukocyte count (TLC), determination of immature/total (*I/T*) neutrophil ratio, platelet count (PC), base excess and random glucose levels. In addition, examination of CSF for protein, glucose, total and differential count, serum electrolytes (Na^+, K^+, Ca^{2+}) etc., was performed, when clinically indicated. Other investigations including chest and abdominal X-rays whenever indicated; in controls, TLC, PC, and CRP were measured.

Case Classification

Cases were broadly classified into two categories, namely "infected" and "non-infected." The infected cases were further sub-grouped into (i) "culture positive sepsis": if culture was positive from any biological source, or (ii) culture negative "clinical sepsis": if 2 or more laboratory parameters were positive, namely TLC > 20,000/μL or <4,000/μL; *I/T* ratio>0.2; PC < 1,00,000/μL; glucose values <45 mg/dL or >180 mg/dL; base excess<-10 mEq/L and CRP>15 mg/L (20). Cases that failed to meet the criteria for the 'infected' group were considered as non-infected. Clinicians remained blinded to all FCM results and the latter did not influence the study classification.

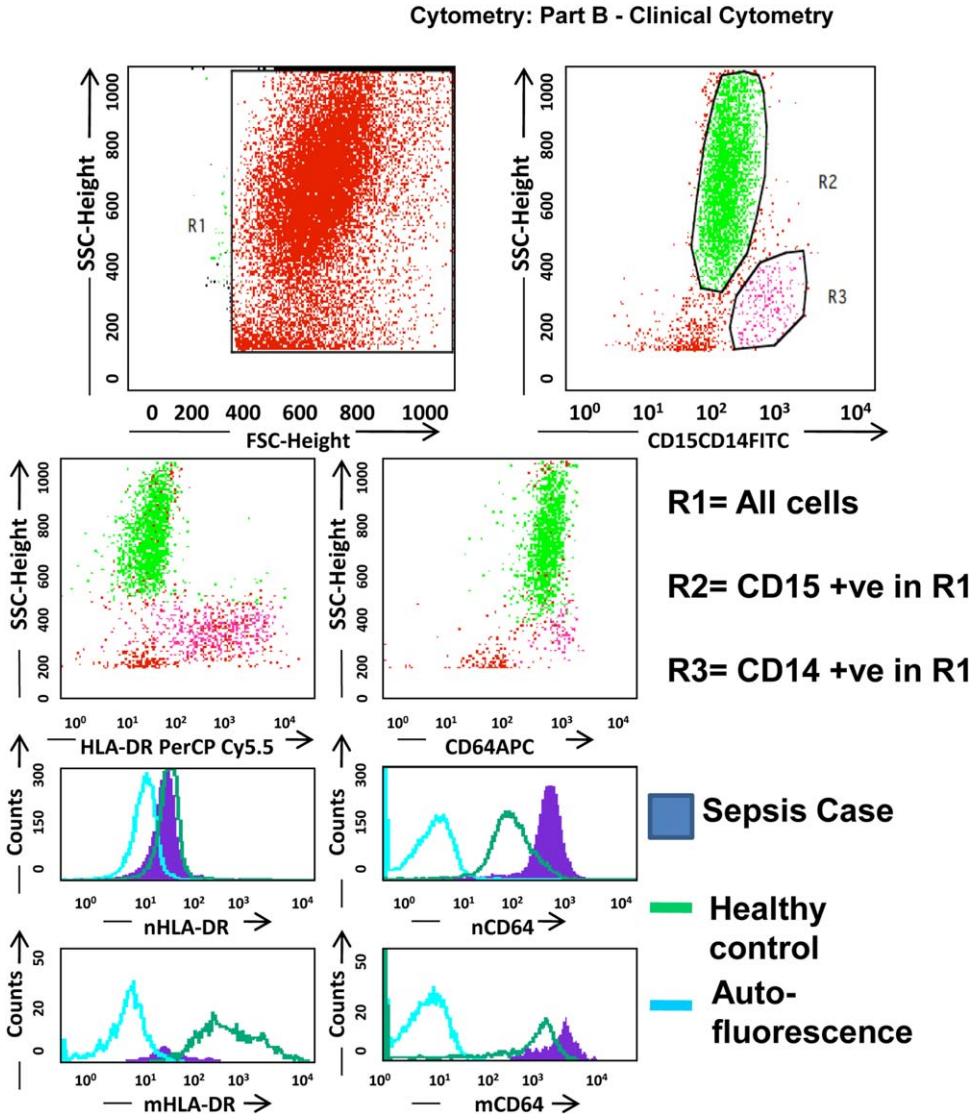

FIG. 1. Immunophenotyping and gating strategy. 20,000 events were collected; Neutrophils and monocytes were gated based on FSC/FITC bivariate dot plot using CD15 and CD14 expression, respectively. Expression was noted in terms of MFI. Neutrophilic CD64 was designated as nCD64 and monocytic mHLA-DR as mHLA-DR. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FCM Analysis

Blood for FCM analysis was collected in EDTA vials and processed within 4 h of collection, except in 2 samples where it was delayed to 6 h. Between collection and processing, the vials were stored at 2–8 °C. During processing, 50 μL blood was added to Tube 1: gating markers, anti-CD15-FITC and anti-CD14-FITC and Tube 2; anti-HLA-DR-PerCPCy5.5, anti-CD64-APC and gating markers. The antibody clones were anti-CD15 antibody (FITC): W6D3; anti-CD14 antibody (FITC): MoP9; anti-CD64 antibody (APC):10.1; anti-HLA-DR antibody (PerCP-Cy5.5): L234. All FCM related reagents were from BD Biosciences (San Jose, CA).

Whole blood was incubated with the antibodies for 15 minutes at room temperature (RT) after which lysis buffer (FACSLyse^{TM}) was added; after further incubation for 15 minutes at RT, 100 μL of distilled water was added; the instrument was calibrated using FACSComp^{TM} beads. The "lyse-no-wash" setting was modified to keep a threshold on the FITC channel and atleast 20,000 events were collected. The gating strategy used to distinguish neutrophils from monocytes relied upon differential side scatter along with CD15 or CD14 expression, respectively. Neutrophils were identified as events with $SSC^{hi}CD15^{hi}$ and monocytes were identified as $SSC^{mid}CD14^{hi}$. Expression of the marker of interest within gated neutrophils and monocytes was measured in terms of median fluorescence intensity (MFI) and analyzed using CellQuestPro software (BD Biosciences, San Jose, CA) (Fig. 1).

Derivation of "Sepsis Index"

Neutrophilic CD64 was designated as nCD64 and monocytic HLA-DR as mHLA-DR. To combine changes in the expression of pro-inflammatory (nCD64) and anti-inflammatory (mHLA-DR) markers, we evaluated a composite parameter by arithmetic division of MFI values of nCD64 and their respective mHLA-DR and multiplying the ratio by 100, and this was termed as "Sepsis Index"

$$\text{Sepsis Index} = \frac{\text{nCD64} \times 100}{\text{mHLA}-\text{DR}}$$

Laboratory Investigations

CRP was measured by nephelometry (BN Prospec® System, Siemens, Germany), detection threshold being 3.19 mg/L. For analyses as a numerical variable, measurements reported as <3.19 mg/L were all assigned a constant value of 3.18 mg/L. TLC and PC was measured in Sysmex 3-part Differential Automated Hematology Analyzers (Sysmex America Inc., Mundelein, IL), base excess was measured in Cobas b 221 Blood Gas system, Roche, Germany and the *I/T* ratio was determined by a pathologist.

Statistical Analysis

Statistical analyses were performed on GraphPad Prism Version 5.0 (GraphPad Software, San Diego, CA, 2007) and SPSS Version 16.0.1 (SPSS, Chicago, IL, 2007). Results are presented as median and interquartile range. For FCM data, in cases where an up-regulation in study parameter was noted, the 90th percentile of controls (excluding outliers) was selected as the diagnostic cut-off; values above this were considered as "positive." On the other hand, for downregulated parameters, 10th percentile of the controls was taken as the cut-off, and values below this were considered "positive." Based on these cut-offs, sensitivity and specificity to detect sepsis was calculated.

All data sets were tested for normality as also paired and unpaired data compared using appropriate statistical tests. To identify the prognostic relevance, survival analysis based on 30-day mortality was performed. To assess the predictability of 30-day mortality by various clinical and laboratory parameters after adjusting for confounders, a binary logistic regression modeling was performed. $P < 0.05$ was considered statistically significant while $P < 0.2$ was considered to show a trend towards significance (21).

RESULTS

Study Population

A total of 106 cases and 29 healthy controls were recruited; incomplete data was available in 6 cases; accordingly, 100 cases were analyzed. The cases were classified into "infected" ($n = 63$) or "non-infected" ($n = 37$). Based on positivity of laboratory parameters, the infected cases were subclassified into culture positive sepsis ($n = 21$) and clinical sepsis ($n = 42$). Demographic characteristics, treatment variables and laboratory parameters are shown in Table 1 and culture yields in Supporting Information 1.

FCM Parameters and Sepsis Index

In the infected group, expression of nCD64 was significantly upregulated while mHLA-DR was significantly downregulated as compared to the non-infected group and healthy controls (Table 2). The Sepsis Index (nCD64 x 100/mHLA-DR) was significantly higher in infected than non-infected neonates and healthy controls (Table 2). The median SI in the infected group was 3.01 fold higher than the non-infected group, *vis-a-vis* a 2.24 fold increase in nCD64 in the infected group as compared to the non-infected group.

Sensitivity and Specificity of Study Parameters to Detect Sepsis

To measure the sensitivity and specificity of SI for detection of infected cases, the 90th percentile of MFI values of healthy controls was considered as the cut-off, and was 92.66. Accordingly, the sensitivity and specificity of SI at Day 1 was 73.01% and 72.22%, respectively (Table 3). Similarly, the cut-off for nCD64 was calculated to be 126.10. The sensitivity and specificity of nCD64 at day 1 was 73.01% and 89.18%, respectively. The cut-off for mHLA-DR was 69.26 (10th percentile of MFI values of healthy controls); the sensitivity for detection of sepsis at day 1 was 25.39% while specificity was 83.33%.

To enhance the sensitivity of detection of sepsis, a combination of parameters were analyzed. Since the sensitivity of mHLA-DR was too low, it was excluded from assessing in combination with other parameters. A combination of nCD64 and/or SI at day 1 increased the sensitivity to 84.12%, an improvement of 11.11% over either alone, indicating only partial overlap between the subsets of sepsis cases detected by individual markers. If positivity of SI and/or positivity of ≥ 2 laboratory criteria was compared with SI alone, the sensitivity at day 1 increased by 14.29%. Similarly, nCD64 when combined with laboratory criteria at day 1, the sensitivity increased by 11.11% (Table 3). A combination of nCD64, SI and positivity of ≥ 2 laboratory criteria increased the sensitivity to 90.47% at day 1. Effect of antimicrobial therapy on the FCM parameters is included in Supporting Information 2.

Prognosticating Survival

At day 1, up-regulation of SI, downregulation of mHLA-DR and a low Apgar score showed a trend towards significance when 30 day non-survivors and survivors amongst infected cases were compared (Table 4). However, it is important to note that the median mHLA-DR amongst non-survivors was greater than the cut-off as decided by the 10th percentile of healthy controls. This essentially means that at least 50% of the non-surviving sepsis cases would have mHLA-DR levels in

Table 1
Baseline Demographic Characteristics of the Study Population

Parameter	Infected	*n*	Non-Infected	*n*	Healthy Controls	*n*	*P* values
Demographic parameters							
Male/Female	40/23	63	21/16	37	16/13	29	0.685
Day of Life (days)	4 (2–11)	63	3 (1.5–5)	37	3 (2.5–4)	29	0.331
Gestational age (weeks)	36 (32–39)	63	35 (32–37)	37	37 (34.5–38)	29	0.137
Birth weight (gm)	2020 (1,241–2,400)	63	2,000 (1,527–2,595)	37	2,197 (1,896–2,376)	29	0.110
Apgar score at 5 minutes	8 (6–8)	63	7 (5–8)	37	8 (7–8)	29	0.094
Treatment and outcome variables							
Duration of stay in NICU (days)	16 (10–26)	63	7 (5–13.5)	37	Not applicable	29	<0.001
Antimicrobial therapy duration (days)	13 (7–18)	63	4 (4–6.5)	37	Not applicable	29	<0.001
30-day mortality (*n*)	13	63	2	37	0	29	0.006
Laboratory parameters on day 1							
CRP (mg/L)	8.55 (3.18–20.90)	63	≤3.19[a]	37	≤3.19[a]	29	NA
TLC (10^3/μL)	10.80 (8.00–19.30)	63	9.40 (7.45–13.25)	37	11.90 (8.00–15.80)	29	0.348
I/T Ratio	0.14 (0.06–0.20)	63	0.08 (0.04–0.11)	37	Not done	–	0.003
PC (10^3/μL)	156.0 (84.0–226.0)	63	224.0 (167.0–295.0)	37	270.0 (198.0–351.5)	29	<0.001[b]
Base excess (mEq/L)	−4.5 (−7.0 to −1.1)	63	−3.2 (−5.15 to −1.75)	37	Not done	–	0.23
Glucose (mg/dL)	82 (65–104)	63	69 (63–83.5)	37	Not done	–	0.056

Data are represented as median (inter-quartile range) or as number of patients, (*n*).
[a]Significant differences between Infected vs. Non-infected and Infected vs. healthy controls in post-hoc analysis.
[b]Detection limit for CRP was ≥3.19 mg/L.

the range of healthy controls; importantly, in case of SI, such an overlap with healthy controls was not observed.

To explore whether a high SI conferred a higher risk of 30-day mortality in sepsis amongst those with infection ($n = 63$, deaths = 13), they were sub-grouped as SI ≤ 92.66 or SI > 92.66; on survival analysis, a high SI (>92.66) showed a trend toward higher mortality ($P = 0.096$), while the majority of deaths (12/13, 92.30%) showed an upregulated SI. The association with mortality was stronger in case of SI, than mHLA-DR< 69.26 ($P = 0.190$), as only 38.4% (5/13) showed a low mHLA-DR (<69.26). SI also showed a stronger association with mortality than nCD64 >126.10 ($P = 0.705$) and CRP >15 mg/L ($P = 0.153$).

For identifying the predictive value of various demographic and laboratory parameters after adjusting for confounders of mortality in neonatal sepsis, binary regression modeling was performed on the infected cases ($n = 63$). Accordingly, parameters that showed a trend toward differences between survivors and non-survivors in Table 4 with a $P < 0.2$ were selected for the predictive model after converting into binary variables. Accordingly, Apgar score (> or ≤ 3) (22), IT ratio (> or ≤ 0.2), PC (<1,00,000/μL or ≥ 1,00,000/μL), mHLA-DR (<69.26 MFI or ≥69.26 MFI) and SI (>92.66 or ≤92.66) consisted of independent variables. On performing a logistic regression, an Apgar score ≤ 3 [odds ratio 11.61 (1.06–127.20), $P = 0.045$] showed the greatest predictive ability while SI >92.66 [odds ratio 4.46 (0.46–42.69), $P = 0.194$] showed a trend towards significance (Table 5). The Nagelkerke R square for the model was 0.305, with an overall correct prediction of 84.10%.

DISCUSSION

Early diagnosis of neonatal sepsis is difficult owing to the non-specific nature of its signs (4), while crucial therapeutic decisions like administration of antimicrobials require strategically timed quantifiable laboratory data. Therefore, the need for an "all-purpose" monitoring marker is paramount to guide clinicians to diagnose and prognosticate mortality early in the disease course; accordingly, this study examined the diagnostic and prognostic role of innate immunity markers in neonatal sepsis, individually and in combination.

Expression of nCD64 is effective in diagnosing neonatal sepsis (23), but its diagnostic efficacy varied from 26–97% (sensitivity) and 71–100% (specificity), possibly due to population heterogeneity, assay methodologies and case classification criteria (23). We considered median of the fluorescence intensity as the reporting parameter, as fluorescence intensity is typically a skewed data. The ROC curve analysis was not used to define the cut-off for nCD64, to allow for comparison of data between neonatal centers. Instead, sensitivity and specificity of parameters and their combinations were calculated based on the 90th percentile of healthy controls for parameters that are upregulated and 10th percentile in case of down regulated parameters. However, had a ROC curve analysis been done, results would have

Table 2
Flow Cytometric Parameters and SI on Day 1

Parameter	Infected	*n*	Non-infected	*n*	Healthy Controls	*n*	*P* values
nCD64 (MFI)	186.00 (117.60–237.10)	63	82.79 (71.46–107.00)	37	79.86 (69.16–111.20)	29	<0.001[a]
mHLA-DR (MFI)	98.22 (66.12–128.60)	63	121.30 (93.28–237.10)	36	154.00 (109.20–213.90)	29	<0.001[a]
SI	173.1 (90.58–333.8)	63	57.40 (30.45–127.80)	36	53.52 (41.27–76.36)	29	<0.001[a]

Data is represented as median (inter-quartile range) or as number of patients (*n*).
[a]Significant differences between Infected vs. Non-infected and Infected vs. healthy controls in post-hoc analysis.

been similar as the diagnostic cut-off by both approaches was similar (Supporting Information Content 3). The diagnostic sensitivity and specificity of nCD64 at day 1 was in concordance with a metanalysis (24) and importantly, the sensitivity and specificity of nCD64 in neonatal sepsis was calculated as defined by EMA guidelines (20), thereby ensuring a standard case definition. Downregulation of mHLA-DR in neonatal sepsis (19,25) was corroborated in our study, but mHLA-DR alone showed a poor diagnostic utility (Table 2).

As the progression of sepsis is a balance between pro and anti-inflammatory responses, phenomena that are not temporally distinct (26,27), capturing the effect of both mechanisms may be an effective approach to monitor sepsis. Pro-inflammatory markers like CRP or nCD64 typically have a protracted rise following pathogenic challenge (28) whereas the anti-inflammatory response like downregulation of mHLA-DR is relatively rapid and more representative of physiological changes (25). Accordingly, a composite parameter, "Sepsis Index (SI)" was derived which when considered alone showed moderate sensitivity and specificity (Table 3); however, in combination with nCD64, the diagnostic sensitivity increased substantially (Table 3), reiterating the importance of using both parameters. At day 1, the best sensitivity of 90.47% was obtained when positivity of nCD64, SI and $\geq$2 EMA criteria were considered (Table 3). The data strongly indicated that when all three parameters are negative at day 1, withdrawal of antimicrobials may be considered. Since the current median number of days of administration of antimicrobials in non-infected cases in our setting was four, this information could obviate 3 days of antimicrobial administration, thus translating into decreased health costs and reduced emergence of antimicrobial resistance.

Various FCM methods can predict prognosis in sepsis episodes (13,17,29) with up-regulation of nCD64 being associated with increased mortality in adults (30,31) while non-survivors showed a down-regulation (32); however, in neonatal sepsis, nCD64 showed no prognostic significance (13). Our study corroborated the same, as in terms of a 30-day survival, nCD64 levels at day 1 showed no prognostic significance. However, mHLA-DR was downregulated in adults and critically ill neonates (14,25) and in adults, was indicative of mortality only at day 3–4 (17,33). Although mHLA-DR was downregulated in non-survivors of sepsis at the very onset, non-survivors were significantly younger than survivors, but no adjustment for multivariate analysis had been done (18), whereas variation of mHLA-DR with postnatal age is documented (25). On the other hand, Ng et al. demonstrated no significant difference in mHLA-DR expression between infected and non-infected or control groups (16). In this study, a trend analysis of mHLA-DR indicated that mHLA-DR was significantly downregulated in non-survivors only at a later stage, i.e., days 4–6 (Supporting Information content 4 and Fig. S1).

On survival analysis at day 1, neonates with higher SI showed a trend towards higher mortality (Fig. 2) and in multivariate analysis, Apgar score $\leq$3 emerged as an important predictor of mortality, concurring with previous reports (34); SI showed a trend towards significance,

Table 3
Sensitivity and Specificity of Markers for Neonatal Sepsis

Parameter with respective cut-offs	*n*	Sensitivity (%)	Specificity (%)
SI> 92.66 on day 1	99	73.01	72.22
nCD64 >126.10 on day 1	100	73.01	89.18
mHLA-DR $\leq$ 69.26 on day 1	99	25.39	83.33
nCD64>126.10 and/or SI >92.66 on day 1	99	84.12	69.44
SI >92.66 and/or $\geq$2 laboratory criteria positive, on day 1	99	87.3	72.22
nCD64 >126.10 and/or $\geq$2 laboratory criteria positive, on day 1	100	84.12	89.18
nCD64>126.10 and/or SI >92.66 and/or $\geq$2 laboratory criteria positive on day 1	99	90.47	69.40

To measure the sensitivity and specificity of nCD64 for detection of infected cases, 90th percentile of the MFI values of 27 neonates (excluding two outliers) was chosen as the cut-off and was calculated to be 126.10. The cut-off for SI was similarly calculated based on the 90th percentile of the SI values from 28 neonates (excluding one outlier) and found to be 92.66. For mHLA-DR, the cut-off of 69.26 was obtained by calculating 10th percentile of the controls (excluding one outlier).

Table 4
Differences in Day 1 Levels Between 30-Day Survivors and Non-survivors

Parameter	Survivors ($n = 50$)	Non-survivors ($n = 13$)	*P* values
Male/Female	31/19	9/4	0.753
Day of Life (days)	4.50 (2.00–11.00)	3.00 (1.50–11.00)	0.467
Gestational age (weeks)	36.00 (32.00–39.00)	36.00 (29.00–38.00)	0.369
Birth weight (gm)	2,010 (1,471–2,370)	2,250 (1,063–2,790)	0.772
Apgar score at 5 minutes	8.00 (6.00–8.00)	6.00 (3.00–8.00)	0.063
CRP(mg/L)	7.14 (3.18–21.15)	15.20 (4.41–52.75)	0.218
TLC(10^3/μL)	10.40 (7.92–16.80)	12.90 (8.20–23.80)	0.312
I/T Ratio	0.14 (0.07–0.21)	0.13 (0.04–0.17)	0.168
PC (10^3/μL)	170.50 (87.75–254.00)	95.00 (70.00–189.00)	0.179
Base Excess (mEq/L)	−3.65 (−6.92 to −0.95)	−6.200 (−9.5 to −1.55)	0.570
Glucose (mg/dL)	81.50 (65.75–103.10)	82.00 (54.50–109.00)	0.513
nCD64 (MFI)	168.50 (116.5–229.8)	211.00 (127.90–274.00)	0.269
mHLA-DR (MFI)	103.20 (70.18–138.60)	69.78 (49.37–104.10)	0.058
SI	164.00 (86.83–312.00)	302.30 (103.3–506.2)	0.066

Data is represented as median (inter-quartile range) or as number of patients.

being much higher than the predictive value of mHLA-DR. As clinical deterioration can be sudden and rapid, the clinician has limited time to intervene, and therefore, a delayed prognosticator will have limited relevance (35). As a high SI showed a trend toward the early prediction of mortality, it deserves exploration in future studies.

An important limitation is the use of CRP as a reference standard and as part of the EMA criteria and the index tests, leaving the chance of bias. However, if such an assessment was performed as in clinical practice, a combination of markers can best detect sepsis. Measurement of Procalcitonin, an emerging marker of sepsis would have been an ideal comparator, but its exorbitant cost precluded the same. Differentiation between monocytes and neutrophils was done using CD14 and CD15, respectively, and although CD14 is mainly expressed on monocytes, the abundant presence of myeloid precursors in a patient with sepsis could cause an overlap with monocytes. However, CD14 expression on neutrophils or myeloid precursors, if present, would be significantly dimmer. It is important however that monocytes and neutrophils are better discriminated, and future studies should keep this in mind. Furthermore, as a single lot of reagents and instrument was used, future studies should aim to standardize the assay between instruments, reagent lots and institutions. In addition, the CD64 clone used was 10.1, which is known to be inferior relative to other clones like 22 and 32.2, although with functionally comparable performances (36); ideally, validation of these clones is necessary. Further, use of a lyse-no-wash staining protocol necessitated triggering the instrument on its FITC channel to exclude RBC debris which resulted in exclusion of lymphocytes from the acquisition gate. However, as lymphocytes can potentially serve as an internal negative control for CD64, exploring a stain-lyse-wash protocol with threshold on the forward scatter channel could be considered. In addition, comparison with validated assays for nCD64 (Trillium Diagnostics) and combination reagents in a single tube should be considered.

In conclusion, our data provides evidence that as an independent parameter, nCD64 is more useful in diagnosis of neonatal sepsis, whereas the SI adds to the overall diagnostic sensitivity at patient admission. In addition, the downregulation of mHLA-DR might help in identification of delayed immunosuppression in neonatal sepsis, and is potentially indicative of a poorer prognosis. At an early stage, a high SI level trends toward greater mortality,

Table 5
Risk Factors and Their Adjusted Odds Ratio for 30 Day-Mortality As Analyzed by Binary Logistic Regression

Independent variable	Adjusted odds ratio (95% CI)	*P* values
Apgar score ≤3	11.61 (1.06–127.20)	0.045
IT ratio >0.2	0.25 (0.02–2.30)	0.222
PC <1,00,000/μL	1.52 (0.36–6.27)	0.563
mHLA-DR (MFI) <69.26	0.94 (0.19–46.78)	0.948
SI > 126.10	4.46 (0.46–42.69)	0.194

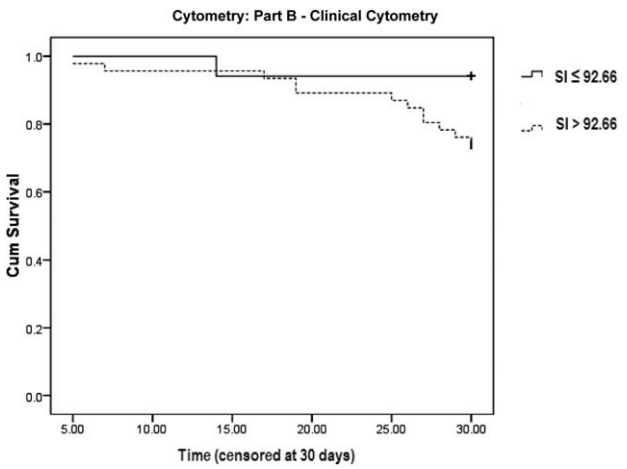

FIG. 2. Kaplan–Meier survival plot stratified for SI below or above the cut-off (92.66 MFI).

but definitive evidence of its advantage over information obtained from nCD64 and mHLA-DR remains to be substantiated.

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