Neutrophil CD64 for Daily Surveillance of Systemic Infection and Necrotizing Enterocolitis in Preterm Infants

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BACKGROUND: Early detection and treatment of infected preterm infants could decrease morbidity and mortality. Neutrophil CD64 has been shown to be an excellent early diagnostic biomarker of late-onset sepsis (LOS) and necrotizing enterocolitis (NEC). We aimed to study whether using CD64 as a daily surveillance biomarker could predict LOS/NEC before clinical manifestation.

METHODS: We collected 0.1 mL whole blood from very low birth weight (VLBW) infants from day 7 postnatal age until routine daily blood tests were no longer required. Four categories of responses were defined: proven sepsis, clinical sepsis, nonsepsis/non-NEC, and asymptomatic CD64 activation.

RESULTS: A total of 146 infants were consecutively recruited and 155 episodes of sepsis evaluation were performed. The biomarker screening utility, sensitivity, specificity, positive predictive value, and negative predictive value for surveillance of LOS/NEC using a cutoff of 5655 antibody-PE (phycoerythrin) molecules bound/cell were 89%, 98%, 41%, and 99.8%, respectively. LOS/NEC was detected a mean of 1.5 days before clinical presentation. However, 63 episodes of CD64 activation occurred in asymptomatic infants who would not otherwise have required sepsis evaluations.

CONCLUSIONS: As a surveillance biomarker, neutrophil CD64 detected LOS/NEC 1.5 days before clinical presentation, but at the expense of performing 41% additional sepsis evaluations. This was mainly attributed to an unexpected group of asymptomatic infants with CD64 activation, who recovered spontaneously and did not require antimicrobial treatment. The latter group has not been previously recognized in VLBW infants and could represent subclinical infection secondary to transient bacterial translocation or mild viral infection.

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Due to the immaturity of their immune systems, preterm, very low birth weight (VLBW)2 infants are vulnerable to life-threatening nosocomial infections and necrotizing enterocolitis (NEC) (1). These conditions remain important causes of morbidity and mortality, despite advances in neonatal intensive care (2). Over the past 2 decades, studies on biomarkers of neonatal sepsis have focused on diagnosing these conditions as early as the time of clinical manifestation (3–9). Although many infection biomarkers have been found to perform satisfactorily during the early phase of illness (at or within 12 h of clinical presentation) (4–6, 9, 10), clinical presentation in infants may occur hours or even days after initial bacterial invasion. Further, because it is now recognized that intense immunoreactive responses associated with systemic infection may affect distant organs, especially the central nervous system (11), it is plausible that commencing appropriate antibiotic treatment even earlier than clinical presentation may achieve better outcomes. Few studies have investigated the use of infection biomarkers as surveillance tools for detection of late-onset sepsis (LOS)/NEC before clinical presentation (12, 13). To qualify as a clinically useful surveillance biomarker, many criteria need to be satisfied, including a very small blood sample requirement to allow daily assays, good agreement of results between venous and capillary blood samples, short laboratory turnaround time, and favorable clinical utility measures (sensitivity, specificity, and predictive values) (14). Studies evaluating surveillance biomarkers have been notoriously difficult to perform and only a few reports are available in the literature.
Neutrophil CD64, a high-affinity IgG Fc receptor, is expressed at low density on the cell surface of nonactivated neutrophils. However, rapid upregulation of the surface antigen occurs upon exposure to bacteria or their components (3, 10, 15). Our previous studies indicated that neutrophil CD64 possessed excellent diagnostic utility for identification of early- and late-onset sepsis and NEC at the time of clinical manifestation of these conditions (4, 7–10, 15). Nonetheless, the surveillance properties of neutrophil CD64 have not been determined. In the current study, we hypothesized that neutrophil CD64 could be used as a surveillance biomarker for very early detection of LOS and NEC in VLBW infants. The objectives were to determine the optimal cutoff value and sensitivity, specificity, and positive and negative predictive values of neutrophil CD64 as a screening tool for early identification of LOS/NEC and to investigate whether daily measurement of neutrophil CD64 can accurately predict these conditions before clinical presentation.

Materials and Methods

PATIENTS
All infants admitted to the neonatal intensive care unit (NICU) at the Prince of Wales Hospital, Hong Kong, with birth weight <1500 g and informed parental consent obtained within 7 days of birth were eligible for enrollment into the study. Infants with major chromosomal abnormalities, lethal congenital malformations, or family history of immunodeficiencies were excluded. Our NICU is a level III unit serving a population of over one million in Hong Kong. The recruitment of VLBW infants was conducted prospectively and consecutively over a period of 39 months.

DAILY NEUTROPHIL CD64 SCREENING
A 0.1-mL sample of whole blood from each recruited infant was transferred to a prechilled EDTA container for neutrophil CD64 evaluation in the morning (0800–1000) starting on day 7 of postnatal age. The blood collection procedure coincided exactly with the daily routine blood tests for hematologic (e.g., complete blood count) and/or biochemistry measurements (e.g., blood gases, electrolytes, renal or liver function tests) so as to minimize sample collection events in these infants. It was also the unit policy to check electrolytes and blood gases on a daily basis for infants on parenteral nutrition and mechanical respiratory support. The blood collection procedure would cease when infants achieved full enteral feeding and no longer required routine monitoring of blood gases.

All samples were immediately transferred to the laboratory in ice bath for quantitative measurement of neutrophil CD64 expression as previously described (10, 15). We incubated 0.05 mL of whole blood with 0.02 mL CD64–phycoerythrin (PE)/CD45–peridinin chlorophyll protein antibodies (Becton Dickinson (BD)) at room temperature for 60 min in the dark. The CD64-PE antibody was of QuantiBRITE grade, of which ≥95% had a PE:antibody ratio of 1:1. The red cells were then lysed with 2 mL of 1× fluorescence-activated cell sorter (FACS) lysing solution (BD) for an additional 60 min before cytometric analysis. Thirty thousand events were acquired for each sample, using the FACSCalibur instrument and CellQuest software (BD). The neutrophil populations were gated according to their CD45/side scatter characteristics, and the expression (geometric mean) of CD64 was measured quantitatively (see Fig. 1 in the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vol59/issue12). There is a strong consensus that for quantitative assays of well-defined cell populations, the use of isotype controls is not recommended (16, 17). In this study, the lymphocyte was used as the negative population to set the threshold for positive CD64 staining (see online Supplemental Fig. 1B). We used QuantiBRITE PE beads which were conjugated to 4 predefined levels of PE molecules for construction of a standard linear regression curve in parallel with each sample analysis. The CD64-PE binding sites per neutrophil were computed and consecutively over a period of 39 months.

CLASSIFICATION OF CLINICAL EPISODES
Four categories of clinical episodes were defined and the classification of episodes into these categories was
translocation or mild viral infection. Presented subclinical sepsis due to transient bacterial infection, or NEC (stage 2 in modified Bell’s classification (18)). Category 2 (clinical sepsis group) consisted of episodes that had been associated with at least 3 clinical features of infection as described in our previous studies (10) and with persistently increased plasma C-reactive protein (CRP) concentrations >10 mg/L for at least 2 consecutive days during the infection period. Although culture results were negative, all clinical sepsis episodes responded promptly to antibiotic treatment, and were not explained by other neonatal illnesses as described in the nonsepsis group. Category 3 (nonsepsis group) consisted of episodes that met the initial clinical criteria for sepsis workup, but the infants were subsequently proven neither to be infected nor to have NEC, and their other parameters, including differential white blood counts and acute-phase proteins, were within the reference intervals. Further, definitive noninfection/non-NEC diagnoses were identified, such as exacerbation of bronchopulmonary dysplasia, apnea of prematurity, gastrointestinal dysmotility of prematurity, heart failure, anemia, or fluctuation of environmental temperature, which could fully explain the clinical manifestations. Category 4 (asymptomatic CD64 activation group) consisted of episodes with sequential up- and downregulation of neutrophil CD64 expression which followed a pattern similar to those of category 1 or 2 infants. However, during such episodes, these patients did not exhibit any clinical signs or symptoms suggestive of LOS/NEC, and thus did not receive sepsis workup or antibiotic therapy. All these patients recovered spontaneously without antimicrobial treatment. It is possible that these episodes represented subclinical sepsis due to transient bacterial translocation or mild viral infection.

STATISTICAL ANALYSIS

We chose the optimal diagnostic cutoff for neutrophil CD64 measurement based on the ROC curve for LOS/NEC while minimizing the number of misclassified episodes. As the diagnostic biomarker should ideally identify all genuine cases of LOS and NEC (i.e., approaching 100% sensitivity) and at the same time would not misclassify too many nonsepsis/non-NEC episodes (i.e., high specificity), the optimal cutoff value was chosen with sensitivity approaching 100% and specificity >85% (14). However, if the biomarker was unable to satisfy the above criteria, then the optimal cutoff value would be chosen so that both sensitivity and specificity approached 85%.

Unlike our previous studies of using biomarkers for diagnosis of systemic bacterial infection and NEC, this study used neutrophil CD64 as a surveillance tool for predicting these conditions. Hence, each daily CD64 result (i.e., each daily time point or patient-day) would be considered an independent clinical indicator to alert frontline neonatologists to perform sepsis workup before signs and symptoms became clinically apparent. This surveillance property of CD64 would be lost once an infection episode had been identified and the biomarker would resume its surveillance role only after an infection episode was over, which in this study was defined as CD64 levels being below the cutoff value for 3 consecutive days. The previously chosen cutoff value was used in calculating the test screening utilities from data taken only on these surveillance patient-days to determine whether CD64 could identify genuine LOS/NEC cases in asymptomatic infants before the onset of clinical presentation.

We used $\chi^2$, Mann–Whitney U, or Kruskal–Wallis tests to compare variables between groups ($\pm$ Dunnnett T$_p$ post hoc correction), for which appropriate. $t$ statistics were calculated to examine whether CD64 values were significantly increased (i.e., more than the diagnostic cutoff) earlier than the clinical presentation. All statistical tests were performed by SPSS (version 18; IBM SPSS). All $P$ values were adjusted with the Benjamini–Hochberg procedure for multiple comparisons.

ETHICS APPROVAL

The study was approved by the Joint Chinese University of Hong Kong and New Territories East Cluster Clinical Research Ethics Committee. Written informed consent was obtained from the parents for all study patients.

Results

A total of 146 of 223 (71.2%) VLBW infants born within the 39-month study period (October 2006 through January 2010) were consecutively recruited into the study. Of 77 nonrecruited infants, 18 were not eligible for recruitment: 15 died within the first week of life, 2 had lethal chromosomal syndromes (Patau and Edwards syndrome), and 1 had multiple congenital abnormalities which were not compatible with long-term survival. Parents of the remaining 59 infants did not consent to the study, either because the research team failed to approach the parents within the first week or because they declined participation. The clinical characteristics of the cohorts for the 4 categories are summarized in Table 1.

There were a total of 155 episodes of sepsis evaluations (Fig. 1), of which 33 and 22 episodes were proven sepsis (category 1) and clinical sepsis (category 2), respectively. The remaining 100 episodes were nonsepsis episodes (category 3). The area under the ROC

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curve (AUROC) was 0.93 (95% CI, 0.88–0.99, \( P < 0.001 \)) and the optimal cutoff value based on the ROC curve was 5655 antibody-PE molecules bound/cell (PE units). On the basis of the episodes of suspected LOS/NEC as defined by infants who had received sepsis evaluation, the diagnostic utilities (95% CI) were calculated. The sensitivities and specificities were 89% (81%–97%) and 95% (91%–99%), respectively (Fig. 1). Using the same cutoff to calculate the screening utilities for LOS/NEC surveillance, the sensitivity, specificity, positive predictive value, and negative predictive value were 89% (95% CI, 81%–97%), 98% (98%–99%), 41% (32%–50%), and 99.8% (99.7%–100.0%), respectively (Fig. 1). LOS/NEC cases were identified a mean (95% CI) 1.5 (1.0 – 2.1) days before clinical presentation (\( P < 0.001 \)). In addition, there were 63 episodes for which CD64 levels were increased in asymptomatic infants who did not require sepsis workup and recovered spontaneously without the need for antimicrobial treatment (i.e., asymptomatic CD64 activation, category 4). Fig. 2 illustrates the patterns of changes in CD64 levels over the course of the episode in the four categories.

There were a total of 6 false-negative episodes classified as category 1 or 2, but the CD64 level remained below the 5655 PE-unit cutoff. Of these, 3 had CD64 levels below the cutoff on the day of clinical presentation but were increased above the cutoff level by the next day. The CD64 levels of the other 3 cases never rose within the next 48 h. Of these latter episodes, 2 fulfilled the criteria of clinical sepsis (category 2) and 1 was a urinary tract infection (category 1, a localized infection).

Table 2 shows the CD64 level profiles of the 4 study categories. There were no significant differences [median (IQR)] in terms of duration of increased CD64 above the cutoff [8.0 (7.0 – 11.0) vs 8.0 (5.0 – 13.5) days, \( P = 1.000 \)], peak CD64 level [23 777 (17 669 – 29 497) vs 19 333 (12 600 – 26 015) PE units, \( P = 0.591 \)] and time to peak CD64 level from baseline [3.0 (2.0 – 4.0) vs 2.0 (2.0 – 4.5) days, \( P = 0.975 \)] between infants in categories 1 and 2, respectively. In contrast, category 4 characteristics were significantly different from category 1 characteristics in terms of duration of increased CD64 [4.0 (3.0 – 6.0) days, \( P = 0.05; \) Fig. 2] and peak CD64 level [8118 (6537 – 11 716) PE units, \( P < 0.001 \); Fig. 2].

Discussion

To our knowledge, this is the largest longitudinal, prospective cohort study using cell surface antigen as a

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**Table 1. Demographic characteristics of the study cohort (n = 146 VLBW infants).**

<table>
<thead>
<tr>
<th>Category</th>
<th>1 (n = 33)</th>
<th>2 (n = 22)</th>
<th>3 (n = 100)</th>
<th>4 (n = 63)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age, weeks</td>
<td>28 (27–30)</td>
<td>28 (27–29)</td>
<td>28 (27–29)</td>
<td>29 (28–30)</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>1005 (825–1240)</td>
<td>1098 (870–1340)</td>
<td>988 (846–1220)</td>
<td>1040 (865–1260)</td>
</tr>
<tr>
<td>Sex, male, n</td>
<td>19 (58%)</td>
<td>13 (59%)</td>
<td>63 (63%)</td>
<td>37 (59%)</td>
</tr>
<tr>
<td>Apgar scores</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 min</td>
<td>7 (6–8)</td>
<td>7 (5–8)</td>
<td>7 (6–8)</td>
<td>7 (6–8)</td>
</tr>
<tr>
<td>5 min</td>
<td>8 (8–8)</td>
<td>8 (7–9)</td>
<td>8 (8–9)</td>
<td>8 (8–9)</td>
</tr>
<tr>
<td>Mother received antenatal corticosteroids, n</td>
<td>31 (94%)</td>
<td>18 (82%)</td>
<td>87 (87%)</td>
<td>56 (89%)</td>
</tr>
<tr>
<td>Mother with chorioamnionitis, n</td>
<td>11 (33%)</td>
<td>6 (27%)</td>
<td>33 (33%)</td>
<td>21 (33%)</td>
</tr>
<tr>
<td>Respiratory distress syndrome, n</td>
<td>31 (94%)</td>
<td>19 (86%)</td>
<td>95 (95%)</td>
<td>60 (95%)</td>
</tr>
<tr>
<td>Oxygen requirement at day 28, n</td>
<td>14 (42%)</td>
<td>11 (50%)</td>
<td>42 (42%)</td>
<td>18 (29%)</td>
</tr>
<tr>
<td>Patent ductus arteriosus, n</td>
<td>5 (15%)</td>
<td>9 (41%)</td>
<td>31 (31%)</td>
<td>14 (22%)</td>
</tr>
<tr>
<td>Intraventricular hemorrhage grade 3–4, n</td>
<td>1 (3%)</td>
<td>2 (9%)</td>
<td>9 (9%)</td>
<td>3 (5%)</td>
</tr>
<tr>
<td>Duration of IPPV/HFOV, days</td>
<td>4 (1–16)</td>
<td>18 (2–36)</td>
<td>4 (1–21)</td>
<td>4 (1–11)</td>
</tr>
<tr>
<td>Duration of nCPAP, days</td>
<td>13 (5–29)</td>
<td>13 (4–23)</td>
<td>13 (4–29)</td>
<td>12 (4–28)</td>
</tr>
<tr>
<td>Duration of parenteral nutrition, days</td>
<td>29 (19–43)</td>
<td>31 (25–44)</td>
<td>20 (13–35)</td>
<td>20 (13–29)</td>
</tr>
<tr>
<td>Age attained full enteral feeding, days</td>
<td>33 (29–45)</td>
<td>37 (28–48)</td>
<td>26 (17–44)</td>
<td>24 (18–33)</td>
</tr>
<tr>
<td>Duration of hospitalization, days</td>
<td>75 (52–110)</td>
<td>98 (70–133)</td>
<td>81 (61–117)</td>
<td>65 (54–101)</td>
</tr>
</tbody>
</table>

*a Values are median (IQR) or n (%). There were no statistical significant differences between groups for any of the demographic characteristics by \( \chi^2 \) or Kruskal-Wallis test with Benjamini-Hochberg adjusted \( P \) value for multiple comparisons.

\( b \) HFOV, high frequency oscillatory ventilation; IPPV, intermittent positive-pressure ventilation; nCPAP, nasal continuous positive airway pressure.
surveillance biomarker for predicting LOS/NEC in asymptomatic high-risk newborns. Our findings suggested that neutrophil CD64 could detect infection in VLBW infants a mean of 1.5 days before clinical presentation using the cutoff of 5655 PE units. However, the advantage of early detection of invasive bacterial infection came at the unexpected expense of the need to perform additional sepsis evaluations (an increase from 155–218, or 41%). We also discovered an unexpected category, the asymptomatic CD64 activation group (category 4), which has never been described in preterm infants. Although the significance of the episodes in category 4 was not fully understood, these cases could represent subclinical infection secondary to transient bacterial translocation or mild viral infection.

There were no significant differences in neutrophil CD64 expression kinetics with regard to the duration of elevation, peak level, and time to reach the peak CD64 level between proven and clinical sepsis groups.

In contrast, the expression kinetics of categories 3 and 4 were distinctly different. For cases ultimately diagnosed as nonsepsis but that initially exhibited some clinical features resembling infection, CD64 levels remained low and did not display a peak. The CD64 expression patterns of category 4 infants were also significantly different from sepsis infants (categories 1 and 2; Fig. 2). In this group of asymptomatic infants, the CD64 profiles were unlikely to have been due to spurious technical or measurement errors, because these cases showed a gradual rise and fall in CD64 activation above the cutoff of 5655 PE units over a 3-day period (Fig. 2). The duration of CD64 elevation and CD64 peak were significantly less than for infants with proven or clinical sepsis. Their case notes were meticulously scrutinized during these episodes. No antibiotics had been given at the time, and the infants remained non-toxic and asymptomatic through the entire episode. It is likely that category 4 episodes represented a host response to minor viral infection or transient bacterial

Fig. 1. A flow diagram demonstrating the steps for evaluation of the optimal CD64 cutoff level and its subsequent application as a screening biomarker for LOS/NEC in asymptomatic infants.
translocation which could be cleared by the infants’ own immune system. As far as we are aware, no previous biomarker studies in neonates have demonstrated similar episodes of upregulation in asymptomatic infants. Further studies to evaluate the causes of asymptomatic activation would be useful to better understand its kinetics and nature. Although it is not possible to prospectively differentiate between asymptomatic CD64 activation cases from those of category 1 and 2, the negative predictive value is sufficiently high to assist clinicians to decide whether to stop antibiotics within 24 h for non-LOS/non-NEC cases.

The strengths of this study are the large cohort size, prospective and longitudinal data collection, and use of a biomarker which can be easily measured in a tiny quantity of whole blood (0.05 mL, equivalent to 1–2 drops of blood from a heel prick). This is in contrast to the majority of other infection biomarker studies for which patients are usually recruited shortly after clinical presentation (9, 12, 13). Thus, diagnostic utilities derived from these studies would also reflect the clinical assessment skills of frontline neonatologists who decided whether or not to recruit the patients. In the current study, patients were recruited prospectively from day 7 of life and followed longitudinally. This provides a unique advantage, because our results reflect solely the ability of neutrophil CD64 to screen for and successfully identify LOS/NEC in asymptomatic infants a mean of 1.5 days before clinical presentation.

Fig. 2. Neutrophil CD64 expression before and after the onset of clinical presentation of LOS/NEC. The optimized cutoff (5655 PE units) is included for reference. For the asymptomatic activation (category 4) episodes, there was no definitive day of clinical presentation, and hence the peak of CD64 elevation was aligned to the CD64 peak of categories 1 and 2.
The main limitation of this study is the lack of a comparative surveillance biomarker. The volume of blood required for measurement of commonly used infection markers such as white cell count (WCC) or CRP (0.5 and 0.6 mL of whole blood in our routine laboratory, respectively) far exceeds the acceptable standard for daily screening in VLBW infants. Because we performed WCC and CRP measurements only in symptomatic infants with clinical features of LOS/NEC, often hours after the onset of symptoms, we could not compare the diagnostic utilities of WCC or CRP with the screening utilities of CD64 in this study, as the latter reflected the ability of the biomarker to detect LOS/NEC in asymptomatic infants before the onset of clinical presentation. We have compared the diagnostic utilities of CD64 against those of CRP in previous studies and found that CD64 was a much better early-warning biomarker for LOS/NEC than CRP. The latter was often upregulated only 8–12 h after the onset of symptoms (10, 15). More importantly, no gold standard biomarker for LOS/NEC surveillance has ever been demonstrated. Further studies with similar design are warranted to investigate whether other biomarkers may be suitable as a screening indicator. Second, we did not include more mature infants in this study. We deliberately selected the VLBW population because they are at the highest risk of LOS/NEC compared with other groups of newborns. Also, it would be unlikely in clinical practice that a surveillance program including all newborn infants would be cost-effective. Further studies in other high-risk children such as immunocompromised or oncology patients should be explored.

In summary, neutrophil CD64 expression used as a surveillance biomarker is both sensitive (89%) and specific (98%) for identifying LOS/NEC in VLBW infants. It can accurately predict systemic infection a mean of 1.5 days before the onset of signs and symptoms. If judiciously used, a LOS/NEC screening program using daily CD64 measurements for high-risk infants may facilitate earlier commencement of antibiotics and other treatments which could potentially improve outcomes of infants with LOS/NEC. However, as with any surveillance program, the disadvantage is the inclusion of false-positive episodes which would translate into increased numbers of sepsis evaluations (an increase of 41%). This study represents the first step in the search for an effective surveillance biomarker of LOS/NEC for preterm newborns. Further studies incorporating the use of additional complementary markers such as the ApoSAA (apolipoprotein serum amyloid A) score (5) in the screening program are warranted for determining whether the benefits of predicting LOS/NEC early can be achieved with a smaller increase in the number of sepsis workups.

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