Effective Biomarkers for Diagnosis of Neonatal Sepsis

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Infection in neonates continues to be a global problem with significant morbidity and mortality. The diagnosis of neonatal sepsis is complicated by nonspecific clinical symptomatology, a high-false negative rate, and a delay in obtaining blood culture results. An ideal biomarker needs to have a high degree of accuracy in recognizing the presence or absence of definite infection at an early stage, to guide the initiation and duration of antibiotic therapy. The diagnostic utility of the following biomarkers seems to be most practical in the early (interleukin [IL]-6, IL-8, tumor necrosis factor-alpha, neutrophil CD64), mid (procalcitonin) and late (C-reactive protein) phases of neonatal sepsis. Future research studies to assess reliability of these biomarkers should be (1) adequately powered for sample size and (2) use the gold-standard definition of blood-culture proven pathogen-specific sepsis. Significant advances in diagnostic accuracy of novel biomarkers to allow early, accurate, and cost-effective identification of pathogens responsible for neonatal sepsis is anticipated in the next 5 years.

Key words. CRP; cytokines; infection; neutrophil CD64; newborn; procalcitonin.

Death due to infections remains a major contributor to mortality in children younger than 5 years of age worldwide [1]. The incidence and etiology of early- and late-onset sepsis in neonates is variable across countries [2–7], which necessitates that antibiotic therapy be tailored in an institution-specific manner. However, the difficulties in confirming the diagnosis of neonatal sepsis have led to the use of a variety of antibiotics for variable durations leading to the emergence of antibiotic-resistant microorganisms [8–12]. Understandably then, there has been significant interest in identification of specific biomarkers of neonatal sepsis [13–15]. In the current context, a biomarker is defined as any measurable parameter that provides meaningful information about the diagnosis of neonatal sepsis. An ideal biomarker for neonatal sepsis would not only have a high degree of accuracy in recognizing the presence (or absence) of definite infection at an early stage, but it would also be useful to guide the duration of antibiotic therapy. Given the risk of delaying antibiotics in neonates with definite sepsis, such a biomarker should be able to make the determination in a reasonably short period (within a few hours). Thus, an ideal biomarker would determine the initiation and length of exposure of antibiotics in a safe manner, decreasing the potential of emergence of resistant microorganisms and mortality in neonates with definite sepsis.

Multiple factors can influence the concentration of a specific biomarker, resulting in variations in the diagnostic accuracy of the same, when compared across studies [16]. Such factors can range from differences in the study population (antenatal/perinatal conditions, gestational age [GA], birth weight [BW], etc), mode and timing of data collection, the nature of the infectious agent causing the inflammatory response in the infant, which, in turn, influences the severity of the presentation and progress of the disease, to the definitions used for “healthy controls” as well as for confirmed/presumed/suspected sepsis. In multicenter studies, comparability of reliability of any single test as a biomarker of neonatal sepsis among institutions may be diminished by variation in disease severity.

Early-onset sepsis (EOS) is usually due to transplacental, ascending, or intrapartum transmission in the perinatal period shortly before or during birth, up to postnatal (PN) day 3 [17, 18]. Late-onset sepsis (LOS) is acquired by horizontal transmission in the home, hospital, or in the community after PN day 4 [17, 18]. By convention, these cutoff values have been used and are reflected in the studies summarized below. Given the problems associated with smaller blood
**Volumes taken from neonates and exposure to antenatal antibiotics (to mention 2 reasons), the chances of getting a positive blood culture (considered the “gold-standard” for diagnosis of sepsis) are markedly reduced. There is no standard definition of clinical sepsis in neonates (which can vary from relying on limited clinical symptomatology to including selective laboratory and radiological investigations), and this inconsistency adds another confounding variable when assessing biomarker studies in neonatal sepsis.**

The search strategy utilized PubMed, focusing on published studies restricted to those in English and after 2008, using the search words “newborn” or “neonatal”, and “sepsis”, and “biomarkers”. Further selection was done to restrict to the most promising and novel biomarkers. Earlier (before 2008) original and review studies were accessed and referred to wherever appropriate. The present review will be mostly limited to studies related to identification and testing of the clinical utility of select biomarkers of neonatal sepsis in the last 5 years, primarily in the neonatal intensive care unit (NICU) population. The focus will be on biomarkers with reporting of details noted above, as available. Most cytokine levels are not expected to be normally distributed in studies done in the neonatal population. Although in some studies the actual cytokine levels have been used to run the analyses, in others receiver operating characteristic (ROC) curves were used to generate the cutoff values. The latter approach will be used in this manuscript, unless otherwise explicitly stated. The primary objective of the present review is to summarize the information about biomarkers that have been reported in “real-life” NICU settings, in order for a list of selected biomarkers to be generated, which would be the focus of further testing in a prospective manner with adequately powered sample sizes using the gold-standard test of positive blood culture as the reference point. The majority of the studies reported in this review have been prospectively done in the NICU setting. Although methodological differences do exist among studies (for example, in the diagnosis of presumed or clinical sepsis) and could account for some of the variability of the results, if a biomarker is going to be potentially clinically useful in the NICU setting, there should be enough of a signal from multiple studies for it to stand out. This method was a practical approach that I believe would narrow down the field to selective biomarkers for investigators to focus their energies upon in the next 5 years.

**Early-Onset Sepsis: Amniotic Fluid**

Given the well known association of presence of infection and inflammation in the intrauterine environment predisposing to EOS to neonatal sepsis, especially those born preterm, access to the amniotic fluid (AF) provides an opportunity to search for biomarkers [19]. Although AF collection is not routinely practiced for detection of EOS in some centers and is associated with the inherent risks (for example, bleeding) of being an invasive procedure, it does allow unique access to the intrauterine environment. Although earlier studies assessed specific cytokines [20, 21], recent investigations into the application of proteomic analyses of the AF have shown significant promise [22]. The Mass Restricted (MR) score was generated from the AF using a single surface-enhanced laser desorption ionization time-of-flight mass spectrometer instrument [22]. Surface-enhanced laser desorption ionization time-of-flight outputs are sequences of values with the molecular weight on the horizontal axis and normalized peak intensity on the vertical axis; the MR score was devised using a stepwise strategy based on filter preferences applied in a sequential manner to result in a numeric score [19]. *A priori* a categorical value of 1 was assigned if a particular peak was present and 0 if absent. Proteomic mapping of the AF revealed a characteristic fingerprint generating a MR score utilizing the presence of 4 biomarkers: neutrophil defensin-1 and defensin-2, along with calgranulins A (S100A8) and C (S100A12). The study specified that a score of 3–4 indicated the presence of inflammation, whereas a score of 0–2 excluded it. Thus, the population was stratified based on the “severity” of inflammation (MR = 0 indicated “no” inflammation; MR = 1–2 indicated “minimal” inflammation; and MR = 3–4 indicated “severe” inflammation) [22]. Women with MR scores of 3–4 were more likely to deliver neonates with EOS [22–24]. These studies were conducted with a prospective collection of consecutive samples, with the neonates being admitted to and monitored in the NICU. Early-onset sepsis was defined as being confirmed (culture positive) or suspected (based on presence of validated hematologic criteria when 2 or more of the following were observed: absolute neutrophil count [ANC] <7500/mL or >14 500/mL, absolute band count [ABC] >1500/mL, immature/total neutrophil ratio [I:T ratio] >0.16, platelet count <150 000 cells/mm³, or an abnormal spinal tap, if done) [22–24]. Neonatal hematological indices and EOS significantly correlated with the MR score, even after adjusting for GA [22]. Although clinicians are likely to treat a symptomatic infant, given a history of clinical chorioamnionitis, if data on AF biomarkers (as noted above) could be made available with a short turn-around time (TAT), it would be an important step towards a more rational use of antibiotics.

It is well known that using traditional culture methods underestimates the infectious etiology of intra-amniotic inflammation (IAI). Towards that end, metagenomic
techniques and sequencing technology for specific identification of species of microorganisms that do not grow in culture, by sequencing 16S ribosomal RNA (rRNA), is now possible [25]. Samples were obtained from a cohort of consecutive patients enrolled at Yale-New Haven Hospital who had paired AF and cord blood available for analysis (n = 161). To avoid selection bias, cases fulfilling the clinical group requirements were selected consecutively based on the availability of at least 1 mL of umbilical vein cord blood for DNA extraction and other research analyses. The study groups were designed using the results of AF and neonatal blood bacterial cultures as reported by the microbiology laboratory. The following groups were studied: Group 1: premature newborns with neonatal blood culture “confirmed” early-onset neonatal sepsis (median GA at delivery, 25 weeks; n = 6); Group 2: premature newborns with negative neonatal blood culture but “presumed” EOS and positive IAI (27 weeks; n = 16); Group 3: premature newborns with negative neonatal blood culture but presumed EOS and negative IAI (32 weeks; n = 7); Group 4: premature newborns without EOS and no IAI (32 weeks; n = 7); Group 5: term healthy newborns (39 weeks; n = 8) delivered by elective Cesarean within the same time period. This last group served as a technical control for handling and analysis of the samples. Six, 15, 5, and 1 patient had histological chorioamnionitis in Groups 1 to 4, respectively, with all 21 samples in Groups 1 to 4, respectively based on the availability of at least 1 mL of umbilical vein cord blood for DNA extraction and other research analyses. The study groups were designed using the results of AF and neonatal blood bacterial cultures as reported by the microbiology laboratory. The following groups were studied: Group 1: premature newborns with neonatal blood culture “confirmed” early-onset neonatal sepsis (median GA at delivery, 25 weeks; n = 6); Group 2: premature newborns with negative neonatal blood culture but “presumed” EOS and positive IAI (27 weeks; n = 16); Group 3: premature newborns with negative neonatal blood culture but presumed EOS and negative IAI (32 weeks; n = 7); Group 4: premature newborns without EOS and no IAI (32 weeks; n = 7); Group 5: term healthy newborns (39 weeks; n = 8) delivered by elective Cesarean within the same time period. This last group served as a technical control for handling and analysis of the samples. Six, 15, 5, and 1 patient had histological chorioamnionitis in Groups 1 to 4, respectively, with all 21 samples in Groups 1 and 2 only having positive results with the culture-independent method from AF or cord blood samples. Five (of 6 in Group 1 only) neonatal blood cultures correlated with positive results with the culture-independent method from AF or cord blood samples. It was noted that 72% of the microbial species identified (Escherichia coli and Fusobacterium nucleatum being the most common) in the cord blood matched those in the AF. Using the 16S rRNA sequencing approach, the paired samples were 99.9%–100% identical [25]. Thus, the strength of this technique to recognize unique microorganisms that are otherwise difficult to detect using conventional culture approaches provides us with the potential for an early diagnosis of EOS [25].

Early-Onset Sepsis: Cord Blood

A variety of cytokines and other acute-phase reactants have been investigated for their potential for biomarkers in umbilical cord blood for diagnosis of EOS [26, 27]. As with multiple previous studies [26], more recent reports [24, 28, 29] have also suggested that cord blood interleukin (IL)-6 and IL-8 seem to have the best discriminatory ability to diagnose EOS using cutoff values derived from area under the curve (AUC) ROC curves (IL-6, 0.86–0.9; IL-8, 0.79–0.87). The last was a prospective case-control study of 120 consecutive preterm infants in a NICU setting; EOS was defined as positive cultures (n = 20) and “highly probable sepsis” (n = 20), using clinical, laboratory criteria (including hematological and C-reactive protein [CRP] values) [29]. Cord blood levels of 2.5–100 pg/mL for IL-6 and 50–300 pg/mL for IL-8 were the range of cutoffs used in the studies reviewed [26, 27]. It is also important to remember that IL-6 levels can be modified by illness severity, including perinatal asphyxia, in noninfected infants [30, 31]. Interleukin-8 levels in cord blood can vary by GA [32]. In addition to IL-6 and IL-8, procalcitonin (PCT) was found to be superior to CRP and tumor necrosis factor-alpha (TNF-α) as diagnostic biomarkers for EOS [27]. The derived cutoff values of PCT used in the cord blood studies ranged from 0.5 to 1.22 ng/mL (AUC, 0.8–0.96) [27]. More importantly, IL-6, IL-8, and PCT levels could be measured in 50–100 μL plasma with TAT of 90 minutes or less [27].

Early-Onset Sepsis: Peripheral Blood

It would be important to mention that whereas cord blood samples are reflective of the intrauterine environment leading to EOS, biomarkers measured in peripheral blood collected at variable time points in the first 3 days of PN life could be potentially influenced by a variety of factors. However, administration of antenatal antibiotics would impact on the incidence of detecting positive blood cultures irrespective of the source of the blood sampling. With the high rate of negative blood cultures usually noted in the classic scenario of EOS, it is imperative that testing of biomarkers be done against the benchmark of the positive blood culture, before being put into clinical practice.

Traditionally, clinicians have relied on using the complete blood count (CBC), despite studies reporting on the unreliability of hematologic indices (namely, total white blood cell count [WBC], ANC, ABC, I:T ratio, and platelet count) for diagnosis of EOS [33]. However, in a retrospective cross-sectional study (n = 67, 623) evaluating the CBC, although there were lower mean values in some of the components of the CBC (WBC and ANC), immature neutrophils were higher in neonates with infection, with no difference in platelet counts [34]. The ability of WBC and ANCs to differentiate the cases of culture-proven EOS did get better with increasing PN age, but these indices were most useful when their values were very low [34]. Based on the above noted findings, the study investigators concluded that the CBC indices were most helpful in detecting EOS after 4 hours; hence, if possible, waiting to initiate
antibiotic therapy after that PN age would be preferred if relying on the CBC results. If there should be significant concern for EOS before that age, antimicrobial therapy should be begun immediately, after sending off the CBC and blood cultures [34].

Earlier studies have suggested that IL-6 derived cutoff levels of 20–50 pg/mL in the peripheral blood of neonates seem to be the most promising marker for neonatal EOS [26], although serial measurements may be necessary for confirmation of infected neonates (prospectively collected samples in a NICU setting with infection diagnosed based on clinical, laboratory, and culture criteria) [35]. A recent prospective study (n = 123; 29 with confirmed/suspected sepsis, 94 with no sepsis) has suggested that using a combination of derived cutoff levels of IL-6 (>250 pg/mL) and PCT (>25 ng/mL) was a reasonable approach to diagnose EOS (culture-proven or “strongly suspected”) [36].

In a recent detailed review of the use of CRP as a biomarker in EOS, it was reiterated that physiologic dynamics (including GA) as well as maternal and perinatal factors can influence the levels of CRP in the first 3 days of PN life [37–40]. C-reactive protein has the best diagnostic accuracy (derived cutoff value of 10 mg/L) when combined with other biomarkers (for example, but not limited to PCT, IL-6, and IL-8) [37]. Interleukin-6 and IL-8 levels can vary by GA and PN age [39, 41]. Serial determination of CRP has been reported to have 99% negative predictive value (NPV) in identifying noninfected neonates, although this should not replace clinical judgment and blood culture results [37]. The magnitude of the CRP response was noted to be higher in Gram-negative versus Gram-positive infections; among the latter, significantly lower median values were reported for coagulase-negative staphylococci [37].

Neutrophil CD64 (nCD64), a high-affinity Fc receptor that increases when neutrophils are exposed to infectious stimuli (primarily secondary to bacterial and fungal agents), has been studied as a marker for EOS. Most studies have reported nCD64 to have good diagnostic accuracy [42, 43]. For blood-culture proven EOS, the CD64 index with a derived cut-point value of 2.38 had sensitivity, specificity, and NPV of 100%, 68%, and 100%, respectively, in a prospective NICU-based study enrolling consecutive infants (n = 580 evaluated for EOS) [43]. This test can be done with a minimal amount of blood (50 μL) with a rapid TAT (~2 hours) in clinical hematological laboratories having dedicated access to flow cytometry [43]. Novel biomarkers with potential usefulness for diagnosis of neonatal EOS have been shown in Table 1.

### Late-Onset Sepsis: Peripheral Blood

Use of the CBC in 1 large retrospective study, which obtained administrative hematological data collected in a prospective manner from 293 NICUs, essentially confirmed that high and low WBC counts, high ANC ratios, and low platelet counts were associated with LOS [44]. Associations were weaker with increasing PN age at the time of the culture. Sensitivities were low for all index cutoffs analyzed, whereas specificity was generally high. The authors concluded that no CBC count index possessed adequate sensitivity to reliably rule out culture-proven LOS in neonates [44].

Most investigators in previous studies have evaluated IL-6 levels in neonates as a marker for LOS [26]. Using the same cutoff value (25 pg/mL) as for cord blood values for EOS seems to have promise in the diagnosis of LOS; however, although the measurements have good sensitivities, the test is not that specific [26]. The clinical usefulness of IL-6 is reduced by its very short half-life, resulting in a rapid decrease in sensitivity [15]. The same disadvantage occurs with IL-8 [45]. Interleukin-6 and IL-8 levels can vary by GA and PN age [39, 41].

Interleukin-6 or TNF-α (both had good sensitivity) combined with CRP (which had good specificity) was a good diagnostic marker for LOS (culture-proven and/or clinical criteria) in a prospective study of preterm neonates [46]. In an independent study median, CRP levels and CBC were useful for the diagnosis of confirmed or probable LOS and comparable with median IL-6 and TNF-α concentrations [47].

As with EOS, most studies have reported nCD64 to be an accurate diagnostic marker for LOS [43, 48]. For

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<th>Table 1. Promising Novel Biomarkers of EOS in Neonates</th>
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<td><strong>Biomarker</strong></td>
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<td>TBARS [29]</td>
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<td>SAA [101]</td>
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<td>nCD64 [43, 81]</td>
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Abbreviations: AF, amniotic fluid; AUC, area under the receiver operating characteristic curve; EOS, early-onset sepsis; MR, mass restricted; nCD64, neutrophil CD64; NPV, negative predictive value; PPV, positive predictive value; SAA, serum amyloid A; TBARS, thiobarbituric acid reactive substances.

*Confirmed or suspected sepsis/total number of infants.

$^3$Calgranulin A, C, Defensin 1, 2.
culture-proven LOS, the CD64 index with a derived cutoff value of 3.62 had sensitivity, specificity and NPV of 75%, 77%, and 96%, respectively, in a prospective NICU-based study enrolling consecutive infants (n = 417, evaluated for LOS) [43]. In a recent study, with a derived cutoff of 2.85, the sensitivity was 87.5% and specificity of 100% for nCD64 index in blood-culture proven LOS [49].

Among noninvasive approaches, using a heart rate characteristics index was shown to be useful for detecting LOS [50]. In a randomized clinical trial utilizing this approach, there was a reduction in mortality from 10.2% to 8.1% (P = .04) in infants who had the heart rate characteristics index visible to clinicians [51]. However, this approach led to 10% more blood cultures being sent and 5% more days on antibiotics in the group with the heart rate characteristics index display [51].

An under-utilized, noninvasive approach is to monitor core-peripheral temperature differences in neonates at risk for sepsis. This has been shown to be a fairly accurate predictor of LOS (culture proven or necropsy findings) in developed [52] as well as in resource-limited countries [53]. There was significant widening of the rectal-sole and axillary-sole temperatures in the preterm neonates with sepsis (culture-proven or clinical or laboratory criteria) (P < .001) [53]. With an overall accuracy of 90.9%, a rectal-sole temperature difference of ≥2.3°C (100% sensitivity) or ≥3.2°C (100% specificity) was a useful marker to differentiate normothermic preterm neonates with or without sepsis. Using the axillary-sole temperature difference, the respective values were ≥2.2°C and ≥3.0°C [53]. These results have been recently replicated in a prospective observational study with proven or probable LOS [54].

Early- and Late-onset Sepsis: Peripheral Blood

Molecular Assays. Using mass spectrophotometric techniques to identify bacteria have the possibility of earlier identification (within 1 hour) of pathogens but modest sensitivities (66%–80%), poor yield when there is low bacterial density, and risk of misidentification of pathogens, especially in situations of polymicrobial growth are significant limitations [55, 56]. The commonly used approach is that of matrix-assisted laser desorption ionization time-of-flight mass spectrophotometry (MALDI-TOF MS). In this process, ionization of a specific sample (in this case, the pathogen that can be detected in a positive blood culture) results in a specific mass spectrum plot (intensity vs mass-to-charge ratio) that determines its identity. Additional details of the sample preparation and the MALDI-TOF MS technique have been published [57, 58].

Molecular techniques to detect the presence of bacterial DNA have been utilized to enhance the diagnostic yield in neonatal sepsis [55, 56, 59]. Polymerase chain reaction (PCR) allows detection of bacterial DNA [55, 56, 59]. Polymerase chain reaction is a technique to amplify a single or a few copies of a DNA strand across multiple orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Approaches to enhance the ability to detect neonatal sepsis include [56]: (1) bacterial DNA amplification from whole blood. In this approach, whole blood is utilized to screen for a highly conserved 16S ribosomal DNA (rDNA) target that is present almost universally in all bacteria but not in human cells. This technique has the advantage of rapid diagnosis but suboptimal sensitivity and specificity. (2) Pre-enrichment of whole blood before target amplification is the next approach. Because increasing the volume of the collected blood sample is not an attractive option in the NICU population, using a tryptic soya broth (TSB) incubation enrichment protocol in conjunction with PCR and pyrosequencing was utilized. In this process, whole blood is incubated with TSB for up to 5 hours before nucleic acid extraction and then screened for bacterial 16S rDNA after the preamplification step. Pyrosequencing determines the order of nucleotides (sequence) in the DNA by detecting the light emitted upon incorporation of the next complementary nucleotide based on the fact that only 1 of 4 (A, T, C, or G) possible nucleotides are added and available at any one given time so that only 1 can be incorporated on the single-stranded template. The percentage of near-term infants with culture-negative results was 98.6% (1216 of 1233), whereas those with PCR-negative results was 97% (1196 of 1233). Compared with blood culture, PCR demonstrated a high NPV (99.2%) and specificity (97.5%). However, the 16S rDNA PCR assay failed to detect 10 of the 17 cases of culture-proven sepsis. (3) Another approach is PCR-based pathogen identification from positive blood culture bottles. In this approach, bacterial DNA is isolated from the specimen, amplified by PCR, detected, and the identity of the pathogen confirmed by hybridization and an alkaline phosphatase reaction on a membrane strip. Others have used a multiplex-PCR-enzyme-linked immunosorbent assay or combined real-time PCR with pyrosequencing. Although studies reported variable sensitivity for pathogen detection (60%–100%) highlighting the challenges of optimizing DNA extraction from small sample volumes, 1 study did show a net gain of 10.7 hours in diagnosing sepsis. (4) The nucleic acid sequence-based amplification (NASBA) approach had sensitivities and specificities ranging from 96% to 100% for bacterial and fungal (Candida) isolates. In the NASBA process, an isothermal nucleic acid amplification assay that preferentially amplifies RNA targets allows rRNA to be expressed at much higher number of
copies compared with PCR. (5) Nonamplification-based fluorescence in situ hybridization (FISH) studies have revealed sensitivities and specificities ranging from 99% to 100% in 2 studies [56]. The FISH approach uses probes that have been developed against specific regions (DNA, RNA) of the targeted pathogen of interest. A meta-analysis of 23 studies utilizing molecular assays for detecting neonatal sepsis has been published [60]. Mean sensitivity and specificity were 90% and 96%, respectively. Real-time PCR and broad-range conventional PCR had higher sensitivity and specificity than other assays. Limitations include need for collection of samples by sterile venipuncture, potential for contamination during collection and processing, need for collection of samples by sterile venipuncture, potential for contamination during collection and processing, need for collection of samples by sterile venipuncture, potential for contamination during collection and processing.

Cytokines and Acute-Phase Reactants. Investigators have noted that TNF-α was the best diagnostic test for sepsis in the NICU (11 with positive cultures, 4 “clinical criteria”) [62], whereas others evaluating IL-6 on the 1st day of symptoms with CRP-positive (measured after 48–72 hours) sepsis cases noted a sensitivity of 77%, specificity of 74%, PPV of 80%, and NPV of 70% [63]. Fungal infections in neonates resulted in significantly higher IL-6 and CRP levels, compared with those with bacterial sepsis [64]. In a NICU study evaluating CRP, IL-6 and immunoglobulin (Ig)M as diagnostic markers for neonatal sepsis (culture-proven or suspected based on clinical and laboratory criteria), it was reported that a CRP of >4 mg/dL gave the best derived cutoff value with 95.7% sensitivity, 88.9% specificity, 78.6% PPV, and 98% NPV [65]. The sensitivity of IgM as a single test at a cutoff derived value of ≥10 mg/dL was 91.3%, with a low specificity of 45% [65]. Likewise, the IL-6-derived cutoff value of 18.2 pg/mL was associated with sensitivity and specificity of 87% and 50%, respectively [65].

C-reactive protein continues to be used as a biomarker for sepsis in multiple studies. It is a late biomarker with a high specificity but poor sensitivity [15, 45]. All studies on severe, invasive bacterial infections in children report higher sensitivities and specificities of PCT than for CRP [66]. C-reactive protein decreases to its normal values after 3–7 days [66]. C-reactive protein levels have been reported to be influenced by GA and noninfectious conditions [67].

Procalcitonin is another extensively studied acute-phase reactant with the advantages of quickly increasing within 4 hours of exposure to bacterial products, peaking at 6–8 hours, and remaining elevated for at least 24 hours [15, 45]. In neonatal bacterial sepsis, plasma PCT concentrations can increase up to 1000 ng/mL, and has been shown to have significant correlations with illness severity as well as survival, but decreases to normal values by day 3 [66, 68]. In addition, compared with CRP, the increase in PCT levels is much faster. Hence, PCT would be preferred over CRP, as a diagnostic biomarker for newborn sepsis [66]. However, the disadvantages of PCT measurements include the fact that reference ranges and thus cutoff values are impacted upon by GA, PN age, and additional clinical conditions [15].

Elevation of PCT levels has been noted up to 48 hours after normal birth. A variety of common neonatal disorders of a noninfectious etiology (ie, perinatal asphyxia, respiratory distress syndrome, and pneumothorax) have PCT concentrations less than [30] or similar [66] to that of neonatal sepsis. Furthermore, ante-, peri-, and postnatal administration of antibiotics can impact on PCT levels [66].

Investigators evaluated PCT, IL-10, and nCD64 as diagnostic markers of EOS and LOS (defined as culture-proven or with clinical signs and CRP >10 mg/L with antibiotic therapy for >10 days) and found that the best combination was of IL-10 (derived cutoff value of >17.3 pg/mL) and nCD64 (cutoff derived value of >2.6%) [69]. The use of IL-6 (derived cutoff value of >21.5 pg/mL) and CRP (derived cutoff value of >5.75 pg/mL) showed moderate accuracy (Q* = 0.79, 0.86, 0.81, 0.82, and 0.77, respectively) [71]. The PCT test was more accurate than the CRP test for the diagnosis of EOS and LOS [71–73]. In a systematic review and meta-analysis of 16 studies (involving 1959 neonates) that utilized PCT (using derived cutoffs ranging from 0.5 to 5.75 ng/mL) as a biomarker for diagnosis of culture-proven or clinical sepsis, the authors concluded that PCT had a higher diagnostic accuracy for LOS than EOS, with the AUC being 0.95 and 0.78, respectively, with an overall AUC of 0.87 [74]. In a recent review, the derived cutoff values of PCT for diagnosis of EOS and LOS ranged from ≥0.5 to >98 ng/mL [68]. Hence, caution must be urged against making firm conclusions given the heterogeneity of the studies included and the lack of uniform definition of sepsis [68, 74].
By activating the complement system, mannose-binding lectin (MBL) results in opsonization of the pathogen; hence, decreased MBL would hamper clearance of microorganisms by the immune system [75]. Among 8 prospective studies evaluated, although 1 study had decreased median MBL levels (170 vs 1450 μg/L), 2 independent studies reported MBL concentrations of ≤400–700 μg/L, in neonates with proven sepsis. The authors concluded that neonates with low MBL levels were associated with culture-confirmed sepsis [75].

In studies that have evaluated both EOS and LOS, nCD64 has been reported to have high sensitivities and NPV [43, 76–81]. Sensitivity NPV of IL-6, CRP, and nCD64 were 80.0%–90.6%, 80.0%–88.8%, and 88.6%–94.0%, respectively, for diagnosis of sepsis (culture-proven or using clinical/validated hematological criteria) in a prospective observational NICU study [79]. Combining all 3 tests increased the sensitivity to 100%; however, specificity and PPV decreased to 62.1% and 55.5%, respectively [79]. Using the database of the largest study done to date on this biomarker in neonatal sepsis [43], a 2.19% nCD64-derived cutoff value for clinical LOS (defined using hematological indices) had sensitivity, specificity, and NPV values of 78%, 59%, and 81%, respectively [81]. The study also reported on derived cutoff values based on BW. For those with a normal BW, it was 2.05, whereas for infants with low BW (LBW <2500 g) and very LBW (<1500 g), the cutoff values were 2.34 and 3.13, respectively [81]. It is likely that differences in the cutoff values for nCD64 in EOS and LOS are reflective of the differences in the predominant microorganisms in each scenario, with the BW categories as a surrogate marker for the maturational aspects of the immune response.

For blood culture-proven sepsis, among the hematological indices, the combination of either the ABC or ANC with nCD64 had the highest sensitivity (91%) and specificity (93%) [81]. Combining nCD64 with PCT provided a sensitivity of 95% and a specificity of 83%, with a NPV of 86% [69]. For further enhancing the sensitivity to 100% for the diagnosis of EOS as well as LOS, it would appear that the combination of nCD64 with specific cytokines (IL-6 or IL-8) and CRP are presently the most promising candidates [81].

In a recent study, use of nCD64 (utilizing a derived cutoff value of 5655 antibody-phycocerythrin molecules bound/cell measurement) for daily surveillance detected LOS/necrotizing enterocolitis 1.5 days before clinical presentation, but at the expense of performing 41% additional sepsis evaluations [82]. Some of the promising novel biomarkers for LOS in neonates have been summarized in Table 2.

### Antibiotic Stewardship

Prompt diagnosis of sepsis and initiation of antibiotic (and appropriate supportive) therapy are critical in the neonatal population because delays can worsen outcomes [83]. It is routine practice in the NICU to start broad-spectrum antibiotics, after a sepsis work-up, while awaiting results of the blood cultures sent [83–85]. Antibiotic use, whether measured in courses or days of antibiotic treatment, was 9- to 14-fold higher in neonates worked up for sepsis compared with those treated for central-line-associated blood stream infections [86]. This increased exposure of antibiotics to neonates in the NICU is not without consequences. Besides separating infants from the parents (while admitted to the NICU), there is also the potential risk for development of antibiotic resistance [87], necrotizing enterocolitis [88–90], LOS [90], prolonged hospitalization with its attendant expenses [91], and increased mortality [90]. Although it has been suggested that serial measurements

### Table 2. Promising Novel Biomarkers of LOS in Neonates

<table>
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<tr>
<th>Biomarker</th>
<th>Sample Size*</th>
<th>Cutoff Value / AUC</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
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<tr>
<td>nCD64 [43, 81]</td>
<td>204 of 533</td>
<td>2.19%–3.62% / 0.73–0.83</td>
<td>75–78</td>
<td>59–77</td>
<td>29–54</td>
<td>81–96</td>
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<tr>
<td>SAA [102]</td>
<td>1231 of 163</td>
<td>&gt; 6.8 mg/dL / 0.71–0.88</td>
<td>44.7–76.4</td>
<td>100</td>
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<td>38.8–58</td>
</tr>
<tr>
<td>ApoSAA1 [103]</td>
<td>42 of 733</td>
<td>0.199</td>
<td>100</td>
<td>61</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>Hepcidin [104]</td>
<td>17 of 44</td>
<td>&gt; 92.2 ng/mL</td>
<td>76</td>
<td>100</td>
<td>100</td>
<td>87</td>
</tr>
<tr>
<td>Calprotectin [105]</td>
<td>62 of 201</td>
<td>1.7 μg/mL</td>
<td>89</td>
<td>96</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>IaIp [106]</td>
<td>45 of 573</td>
<td>&lt; 177 mg/L / 0.94</td>
<td>89.5</td>
<td>99</td>
<td>95</td>
<td>98</td>
</tr>
<tr>
<td>CD11b [107]</td>
<td>65 of 77</td>
<td>290 fluorescent units</td>
<td>75</td>
<td>100</td>
<td>100</td>
<td>86</td>
</tr>
</tbody>
</table>

Abbreviations: AUC, area under the receiver operating characteristic curve; CD11b, cell surface marker on neutrophils; EOS, early-onset sepsis; IaIp, interalpha inhibitory proteins; LOS, late-onset sepsis; nCD64, neutrophil CD64; NPV, negative predictive value; PPV, positive predictive value; SAA, serum amyloid A.

*Includes 10 infants with EOS.

†Confirmed or suspected sepsis/total number of infants.

‡Includes 10 infants with EOS.

§LOS and necrotizing enterocolitis used as a composite outcome.
of CRP may dictate the duration of antimicrobial therapy in the NICU [15, 45], it has not yet been proven to do so [66].

Among the most promising biomarkers of neonatal sepsis that have strong potential to reduce unnecessary antibiotic use are PCT and nCD64. Use of PCT has been shown to decrease the duration of antimicrobial treatment by 2–4 days, in a meta-analysis of randomized controlled trials in intensive care units, which did include neonates (n = 121), with no apparent adverse clinical outcomes [92]. A pilot study using PCT to guide treatment has reported a significant absolute risk reduction of 27% (P = .002) in neonates treated for ≥72 hours, with an average decrease of 22.4 hours for the duration of antibiotic therapy [93]. A multicenter, randomized trial on the efficacy and safety of PCT-guided treatment in neonates is ongoing (clinicaltrials.gov: NCT00854932) [66].

As regards nCD64, its high NPV and decrease in concentration on sequential measurements in blood culture-positive cases on treatment suggest the potential for its use in deciding the initiation and duration of antibiotic use [43, 76, 81]. Currently, a single-center randomized controlled trial using sequential nCD64 concentrations for decision making to stop antibiotics early in EOS and LOS in neonates is planned (clinicaltrials.gov: NCT01825421).

Expert Commentary

From the practical viewpoint, 1 approach would be to categorize the biomarkers of neonatal sepsis based on the detection time from the perspective of point-of-care diagnostics into early, mid, and late phases [14]. Defining the phase is complicated by the fact that it is dependent upon when the sample was collected and analyzed during the course of the neonatal sepsis. Thus, the “start point” could be the time when the clinical suspicion of sepsis was initially triggered or when the neonate was first brought to the attention of the medical team. In the latter scenario, this may not coincide with the onset of illness and could be extremely variable. The timings suggested are based on experimental animal data and sequential measurements conducted in closely monitored settings (ie, NICUs). Promising early phase (2–12 hours) biomarkers would be IL-6, IL-8, nCD64, and TNF-α, whereas mid phase (12–24 hours) would be PCT, and the late phase (>24 h) being CRP [14]. Based on the data summarized above, diagnostic utility of the following biomarkers would be most practical in the clinical setting: IL-6, IL-8, TNF-α, nCD64, PCT, and CRP. These have been summarized in Table 3. The availability of daily testing and TAT in clinical laboratories of the listed biomarkers would be somewhat dependent on the institutional resources, but most centers with Level IV NICUs would be expected to have access to at least 2 or more of the tests (specifically, nCD64, PCT, and CRP). Regarding the cytokines, a few caveats need to be considered. First, despite a theoretical TAT ranging 1.5–6 hours, cytokines are not commonly measured in a stat laboratory, and therefore their clinical use in an emergency setting is unreliable, at least in more than 90% of all clinical laboratories. Second, cytokines measurement accuracy is influenced by the preanalytical phase: cytokines should be measured after short time from blood collection; the blood sample should be collected in tubes containing specific anticoagulants, and the temperature of sample transport transportation also influences the stability of these molecules.

Among biomarkers of sepsis for which limited or no information is available on neonates, but based on research studies conducted on adults and children, those that would have strong potential to be clinically useful are angiopoietin 2, haptoglobin, soluble triggering receptor expressed on myeloid cell (sTREM) and soluble urokinase plasminogen activator receptor (uPAR) [15, 49, 94–100].

It is imperative that future studies focused on establishing reliable biomarkers for diagnosis of neonatal sepsis should not only be adequately powered for sample size but also use the gold-standard definition of blood-culture proven pathogen-specific sepsis. The use of minimal

<table>
<thead>
<tr>
<th>Phase</th>
<th>Biomarker</th>
<th>Cutoff Values</th>
<th>Blood/Serum/Plasma Volume Required</th>
<th>Turnaround Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early (2–12 h)</td>
<td>IL-6</td>
<td>10–150 pg/mL</td>
<td>2–100 μL</td>
<td>1.5–6 h</td>
</tr>
<tr>
<td></td>
<td>IL-8</td>
<td>60–300 pg/mL</td>
<td>2–50 μL</td>
<td>1.5–6 h</td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>12–90 pg/mL</td>
<td>2–200 μL</td>
<td>1.5–6 h</td>
</tr>
<tr>
<td></td>
<td>nCD64</td>
<td>2.4–3.6 μL</td>
<td>50 μL</td>
<td>1–2 h</td>
</tr>
<tr>
<td>Mid (12–24 h)</td>
<td>PCT</td>
<td>0.5–5.75 ng/mL</td>
<td>20–200 μL</td>
<td>20 min–5 h</td>
</tr>
<tr>
<td>Late (&gt;24 h)</td>
<td>CRP</td>
<td>4–10 mg/L</td>
<td>5–20 μL</td>
<td>1–4 h</td>
</tr>
</tbody>
</table>

Abbreviations: CRP, C-reactive protein; IL, interleukin; LOS, late-onset sepsis; nCD64, neutrophil CD64; PCT, procalcitonin; TNF, tumor necrosis factor.

*See text for more details.

†For EOS.

‡For LOS.
blood volumes for sample collection and quick TAT for diagnostic tests are always critical factors for the neonatal population. In addition, the utility of serial measurements of such biomarkers should be assessed for antibiotic stewardship in the neonatal population.

Five-Year View

One would anticipate that given the strength of the evidence noted in the preceding pages, nCD64 and PCT would emerge as the best combination of biomarkers for diagnostic accuracy of neonatal EOS and LOS as well as for deciding upon the initiation and duration of antibiotic therapy. In nurseries where the access to the above resources is limited or unavailable, CRP measurements would be preferred or continue to be used for the same purposes.

In terms of newer biomarkers, those listed in Tables 1 and 2 would have additional data generated that would help sort out if they actually are going to be useful as diagnostic markers for neonatal sepsis. Among those listed as having little or no neonatal data available, angiopoietin 2 would be the one most aggressively pursued given the experimental data supporting a significant role in the pathogenesis of sepsis and the wealth of data reporting it to be a promising biomarker for sepsis in adults and children. In addition, one would expect significant advances to be made in the molecular assays for diagnosing EOS and LOS, with further refinements to allow early, accurate, and cost-effective identification of pathogens responsible for neonatal sepsis.

Key Issues

- The diagnosis of sepsis in neonates is complicated by nonspecific clinical symptomatology, a high false negative rate, and a delay in obtaining positive blood culture results.
- An ideal biomarker for neonatal sepsis needs to have a high degree of accuracy in recognizing the presence (or absence) of definite infection at an early stage, with results being available with a short TAT, for it to be useful to guide the initiation and duration of antibiotic therapy.
- Amniotic fluid, cord and peripheral blood have been used as sources to detect biomarkers for neonatal sepsis.
- Promising early-phase biomarkers would be IL-6, IL-8, nCD64, TNF-α, whereas mid phase would be PCT, and the late phase being CRP.
- Among noninvasive biomarkers for neonatal sepsis, the heart rate characteristics index and core-peripheral temperature differences show promise.
- Novel biomarkers of neonatal sepsis that require additional research include MR score, haptoglobin, serum amyloid A, hepcidin, interalpha inhibitory proteins (Iaip), MBL, angiopoietin 2, sTREM, and soluble uPAR.
- The utility of serial measurements of such biomarkers should be assessed for antibiotic stewardship in newborns.
- Refinements to allow early, accurate, and cost-effective identification of pathogens responsible for neonatal sepsis would be anticipated in the next 5 years.

Acknowledgments

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Author has submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

References

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