Screening and Serial Neutrophil Counts Do Not Contribute to the Recognition or Diagnosis of Late-Onset Neonatal Sepsis

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Objective To determine the validity of screening and serial neutrophil counts in predicting the absence/presence of late-onset sepsis (LOS) in infants with central venous catheters.

Study design Retrospective study of infants admitted to the neonatal intensive care unit (2009-2013) at Parkland Hospital with a central venous catheter and ≥1 LOS evaluations. Infants were categorized as proven or suspect LOS or uninfected based on results of blood cultures, clinical illness, and duration of antibiotics. Receiver operating curves (ROCs) were constructed to predict the absence or presence of LOS using Manroe reference ranges for total and immature neutrophils and the immature to total neutrophil ratio at 0, 12, and 24 hours after blood culture and the neutrophil value score, which assesses serial values.

Results Of the 497 infants with a central venous catheter, 179 underwent ≥1 LOS evaluations, and 140 of 179 (78%) had ≥1 complete evaluations (2 blood cultures and neutrophil values at 0, 12, and 24 hours), resulting in 188 complete LOS evaluations. The gestational age was 28 ± 4 weeks and LOS evaluation occurred at 29 ± 34 days (SD; 4-197 days). Sixty-one (35%) infants had proven LOS, 48 (23%) were suspect, and 71 (38%) were noninfected. ROC comparing proven vs noninfected was ≤0.56 for total neutrophils, immature neutrophils, and immature to total neutrophil ratio at 0, 12, and 24 hours and similar for proven + suspect vs noninfected. ROC for neutrophil value scores and absence of LOS was 0.56.

Conclusions Screening neutrophil values are poor predictors of LOS in neonates with a central venous catheter, as are serial neutrophils and the neutrophil value score. Alternative biomarkers are needed. *(J Pediatr 2019;205:105-11).*

The incidence of neonatal late-onset sepsis (LOS), ie, systemic infection >72 hours after birth, has decreased in recent years.[1] Nonetheless, many infants born preterm undergo LOS evaluations followed by prolonged antibiotic therapy in the absence of positive cultures due to concern for adverse long-term outcomes.[2-6] This is particularly true in infants with a central venous catheter.[3,7] Although blood cultures are the gold standard for the diagnosis of LOS, they may be negative when clinical signs suggest LOS. Biomarkers are needed to accurately identify infants who should or should not receive antibiotic therapy. In the absence of valid biomarkers or a positive blood culture, infants may have excessive antibiotic exposure, which can be associated with the emergence of resistant organisms, increases in fungal infections, or alterations in the gastrointestinal microbiome.[2,6,8,9]

The complete white blood cell count (CBC), in particular neutrophil values from the differential cell count, is used extensively to recognize or diagnose LOS.[2,10] Manroe et al established reference ranges (RRs) for neonatal neutrophils from birth to 30 postnatal days and introduced the absolute neutrophil values and immature/total neutrophil ratios (I:T), a measure of the "left shift," in assessing neonatal sepsis within 72 hours of birth.[10,11] Mikhael et al recently validated the RRs and demonstrated their utility in predicting the absence of early-onset sepsis (EOS).[12] However, the positive and negative predictive values for LOS are unclear.[13-15] The purpose of this study was to assess the use of neutrophil values from the manual cell count in the diagnosis of LOS in infants with a central venous catheter and at high risk for LOS using published RRs.[11] A secondary purpose was to compare the distribution of neutrophil values in the infants who were not infected with the RR previously reported by Manroe et al.[11]

ATI	Absolute total immature neutrophil count
ATN	Absolute total neutrophil count
AUC	Area under the curve
CBC	Complete white blood cell count
EOS	Early-onset sepsis
I:T	Immature to total neutrophil ratio
LOS	Late-onset sepsis
NICU	Neonatal intensive care unit
ROC	Receiver operating curve
RR	Reference range

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Funded by George L. MacGregor Professorship (to C.R.). The authors declare no conflicts of interest.

Portions of this study were presented at the Pediatric Academic Societies annual meeting, May 6-9, 2017, San Francisco, California.

https://doi.org10.1016/j.jpeds.2018.09.024

Methods

This was a retrospective, observational study of all neonates admitted to the Parkland Hospital Neonatal Intensive Care Unit (NICU), Dallas, Texas, between April 2009 and January 2013. This coincided with the establishment of a central venous catheter database that would allow study of the incidence of infection and facilitate study of infants who were infected. This is a level IIIC NICU with approximately 51 864 deliveries and 4611 admissions during the study period. Infants were included if they had a central venous catheter, inserted either surgically or peripherally, and underwent a complete LOS evaluation >72 hours after birth based on clinical signs (eg, respiratory distress, seizures, apnea, or hypoglycemia) and received antibiotic therapy. A LOS evaluation was considered complete if it included 2 blood cultures, one from the central venous catheter and a second from a peripheral site, serial CBCs with manual differential white blood cell counts obtained at initiation of LOS evaluation (0 hours) and 12 (10-16 hours) and 24 hours (22-28 hours) later, and the collection of urine and spinal fluid cultures.

Infant LOS evaluations were categorized as follows: proven if 1-2 blood cultures were positive at ≤48 hours and infants received at least 5 days of antibiotic therapy; suspect if both blood cultures were negative by 48 hours or positive after 48 hours, but infants were considered clinically ill and received at least 5 days of antibiotic therapy, and noninfected if blood cultures were negative by 48 hours or positive after 48 hours and considered a contaminate, the infant was clinically well, and antimicrobial therapy was <72 hours. Neutrophil data from noninfected infants (n = 71) were used to determine whether they were distributed within the upper and/or lower cutoffs described in the Manroe RRs as recently reported by Mikhael et al.[11,12] Additional clinical data were collected from the electronic medical record and an existing validated database that included all admissions to the NICU for >30 years, including gestational age, birth weight, race/ethnicity, neonatal complications, and clinical diagnoses. The study was approved by the institutional review boards of the University of Texas Southwestern Medical School and Parkland Health and Hospital Systems.

The differential cell count was determined manually as previously described.[10-12] The absolute neutrophil values were calculated from the corrected white blood cell count, which removes nucleated red blood cells. Each differential was based on the manual count of 200 cells. The manual absolute total immature neutrophil count (ATI) value includes bands, promyelocytes, and metamyelocytes.[11,12]

Statistical Analyses

Demographics and clinical variables for infants within each group as well as neutrophil values were compared with an ANOVA and the χ^2 test. A Bonferroni correction was used in pair-wise comparisons of the 3 groups when ANOVA was $P \leq .05$. Receiver operating curves (ROCs) were constructed for abnormal neutrophil values based on the Manroe RR at each time period when a CBC was obtained to identify neutrophil values and time periods with the best predictability for the presence or absence of LOS.[12] Serial neutrophil values, ie, the 3 sets of values obtained at 0, 12, and 24 hours, were assessed using the neutrophil value score described by Mikhael et al for EOS.[12] This consists of determining the sum of absolute total neutrophil count (ATN), ATI, and I:T values "within" the upper and lower limits of each RR, resulting in a maximum score of 9. In EOS, if 6-8 of the 9 values were within the RR, the specificity was 100% or area under the curve (AUC) >0.80 for the prediction of no infection. ROC curves were constructed to assess the neutrophil value score in determining the absence of LOS. Data are presented as mean ± 1 SD unless otherwise noted.

Results

During the study period, 4611 neonates were admitted to the NICU, and 497 (11%) had a central venous catheter placed during their hospitalization; 179 of 497 (36%) underwent at least 1 LOS evaluation. Of those with an LOS evaluation, 140 infants (78%) had ≥1 complete evaluations that included 3 serial CBCs and 2 blood cultures from different sites, resulting in 188 complete LOS evaluations. Thirty-three infants within the 3 treatment groups had >1 LOS evaluation; as a group they underwent 2.3 evaluations per infant. Fifty-four infants were categorized as proven LOS, 32 as suspect, and 46 as noninfected. Eight infants were considered to have had contaminants in their blood culture, ie, the blood culture was not positive until >48 hours and antibiotic therapy was <5 days, and were excluded from subsequent analyses. There were no significant differences across groups in gestational age, birth weight, race, sex, or age at LOS evaluation (**Table I**). As anticipated, there were group differences in the duration of antibiotic therapy received. The results were not different analyzing means vs medians.

Cultures

Within the 61 proven LOS evaluations, 43 (70%) grew coagulase-negative *Staphylococcus* in the blood culture with the time to first detection 27 ± 11 hours (7-47 hours), 9 grew other gram-positive organisms with a time to detection 20 ± 9 hours (12-38 hours), and 9 (14.8%) grew gram-negative organisms with a time to first detection 13 ± 4 hours (8-23 hours), which was significantly earlier than coagulase-negative *Staphylococcus* ($P < .01$, ANOVA). When the site of a positive blood culture was examined, 25% were only from the central venous catheter, 25% only from a peripheral site, and 50% from both sites, demonstrating the utility of obtaining blood cultures from 2 sites. When gram-negative organisms were involved, 67% (6/9) were positive in both sites vs 42% (18/43) for episodes of gram-positive organisms. The site did not change the time to first detection. There were no positive spinal fluid cultures or evidence of fungal infections. Five episodes of proven infection were associated with a positive urine culture, but the organism always differed from the blood culture. Four episodes of suspect infection (8.3%) and 2 episodes within the noninfected had a positive urine culture.

Table I. Clinical characteristics of infants with a central venous catheter who underwent ≥1 complete evaluations for LOS

Variables	Total (n = 140)	Proven (n = 54)	Suspect (n = 32)	Noninfected (n = 46)	Contaminate (n = 8)
Gestational age, wk	28.3 ± 4†	28.9 ± 4	27.2 ± 3	28.2 ± 3	29.0 ± 3
	28 (26, 30)‡	28 (26, 31)	27 (25, 28)	28 (26, 30)	28 (28, 29)
Birth weight, g	1131 ± 56	1215 ± 5	1050 ± 60	1076 ± 480	1216 ± 636
	940 (770, 1260)	1000 (788, 1469)	890 (753, 1068)	980 (785, 1281)	935 (845, 1653)
Male	59 (42)*	23 (43)	16 (50)	20 (44)	0 (0)
Race/ethnicity					
Hispanic	57 (41)	17 (32)	10 (31)	27 (59)	3 (38)
African American	35 (25)	16 (30)	10 (31)	8 (17)	1 (13)
White	45 (32)	20 (37)	11 (34)	10 (22)	4 (50)
Other	3 (2)	1 (2)	1 (3)	1 (2)	0 (0)
Number LOS evaluations	n = 188	n = 61	n = 48	n = 71	n = 8
Postnatal age at LOS, d	29.2 ± 34	34.8 ± 40	30.2 ± 39	24.2 ± 25	25.0 ± 11
	18 (11, 28)	19 (13, 34)	17 (11, 28)	17 (11, 26)	26 (15, 32)
Duration of antibiotics, h	134 ± 75	180 ± 52[a]	186 ± 71[a]	65.2 ± 20[b]	78.0 ± 17[b]
	144 (72, 168)	168 (168, 180)	168 (144, 168)	72 (48, 72)	72 (72, 96)

*Values in parenthesis are percent of population in the column. Data were analyzed by the Kruskal–Wallis test and ANOVA across rows; different superscripts across rows denote significant differences by Bonferroni post-hoc tests, $P < .01$. Absence of superscripts denotes no differences across rows.
†Means ± SD.
‡Medians (25th, 75th percentile).

Distribution of Neutrophil Values

Two hundred thirteen neutrophil counts were available from the 71 noninfected episodes and were plotted on the appropriate RR of Manroe et al at >72 hours of age to determine whether as a group their distribution differed from the original RRs.[11] Sixty-three percent of noninfected ATN values exceeded the upper cutoff (**Figure 1**, A; available at www.jpeds.com) and demonstrated a nonspecific neutrophilia (χ^2 test $P < .01$); only 3% were below the lower cutoff (1800 cells/mm^3) and did not differ from expected (χ^2 test $P = .8$). In addition, 30% of ATI and 17% of I:T values (**Figure 1**, B and C; available at www.jpeds.com) exceeded the cutoff, ie, >500 cells/mm^3 and >0.12, respectively (χ^2 test $P < .01$). We also compared the distribution of the ATN, ATI, and I:T between proven, suspect, and noninfected episodes. There were no differences between groups in the distribution of ATN and ATI values (χ^2 test $P > .05$; data not shown), demonstrating substantial overlap; however, the distribution of I:T values differed between proven and noninfected episodes, ie, 83% vs 74%, respectively, exceeded 0.12, χ^2 test $P = .03$.

Prediction of LOS

To determine the predictability of each neutrophil value for the presence or absence of LOS, we calculated the sensitivity, specificity, and positive and negative predictive values for proven and proven + suspect vs noninfected episodes using the Manroe RR and cutoffs (**Table II**). Except for the specificity of the I:T proportion, the predictive values, eg, sensitivity and positive predictive value, for each neutrophil value were ≤71%. Although the specificity of the I:T was 83% for proven and proven + suspect, the negative predictive values were <52%.

There were 1620 neutrophil values available from the 3 treatment groups that were collected at initiation of the LOS evaluation and 12 and 24 hours later. To determine whether the neutrophil values accurately predicted the presence of LOS, ROCs were constructed for each time period, ie, 0, 12, and 24 hours, and each neutrophil value to determine the AUC for ATN, ITN, and I:T values, comparing proven (**Figure 2**, A) and proven + suspect LOS (**Figure 2**, B) vs noninfected irrespective of the infecting organism. No single neutrophil value at any time point had an AUC >0.54, demonstrating moderate-to-poor predictability for the presence of LOS. This also included the initial CBC values that might be considered a "screening" CBC.

Because neutrophil responses might differ according to the organism detected,[8] organism-specific ROCs were constructed within the proven LOS group. The AUCs for all gram-positive organisms for all neutrophilic values were <0.65 (**Figure 3**, A; available at www.jpeds.com). Although the AUC for the initial I:T in the presence of a gram negative organism was 0.84 (**Figure 3**, B; available at www.jpeds.com), it was <0.6 at 12 and

Table II. Prediction of LOS using individual neutrophil variables in neonates with proven and proven plus suspect LOS

Neutrophil values	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Proven*				
ATN (≥5400 and ≤1800)	68	34	52	50
ATI (≥640)	31	71	53	50
I:T (≥0.12)	27	83	63	52
Proven plus suspect*				
ATN (≥5400 and ≤1800)	67	34	63	38
ATI (≥640)	33	71	65	39
I:T (≥0.12)	25	83	71	40

NPV, negative predictive value; *PPV*, positive predictive value.
*Threshold cutoffs included for sensitivity, specificity, and PPV and NPV are in parentheses and are derived from the Manroe RRs >96 hours postnatal.[11]

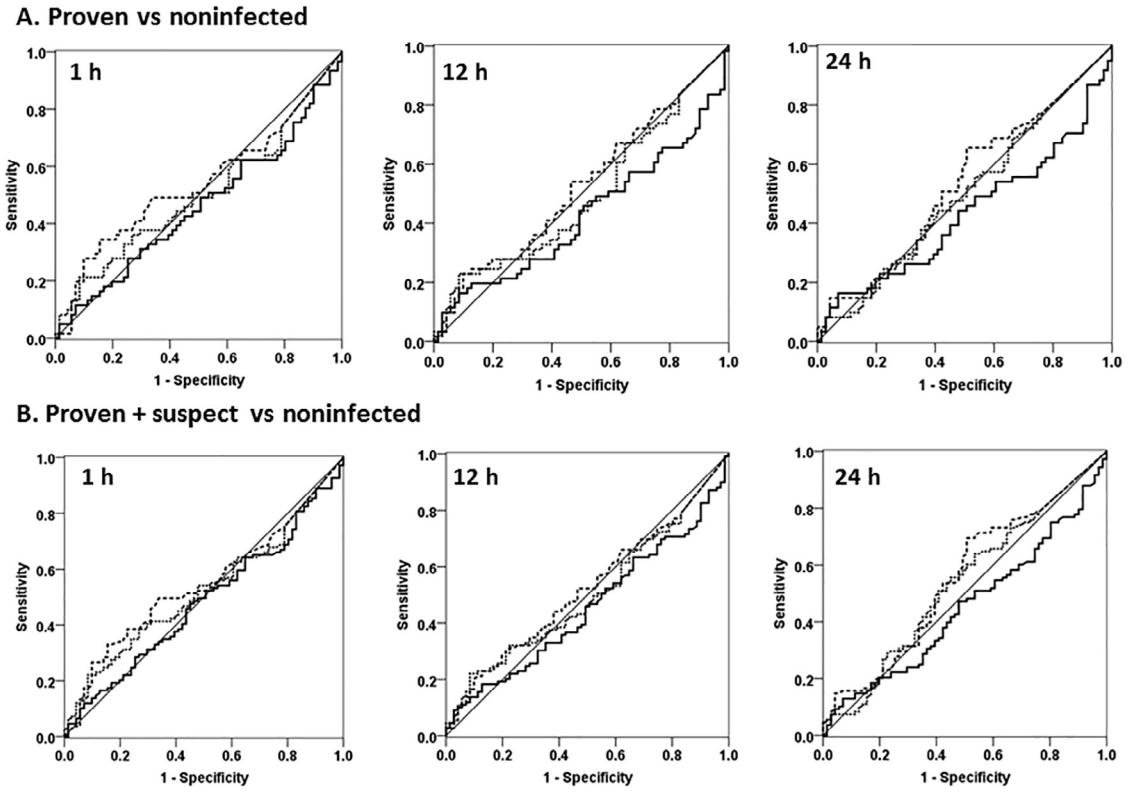

Figure 2. ROC curves for ATN (*solid lines*), ATI (*dotted lines*), and the I:T (*dashed lines*) at the time of initiating an evaluation for LOS and at 12 and 24 hours later comparing **A,** proven and **B,** proven + suspect vs noninfected. The *diagonal line* represents the line of identity where ties are encountered.

24 hours; moreover, the AUCs for all ATN and ITN values were <0.64.

We examined the use of the neutrophil value score, using the 9 serial neutrophil values obtained and evaluating the number of values within the RR for each infant. The AUC for the neutrophil value score was ≤0.56 for both of the ROCs comparing noninfected vs proven and proven + suspect (**Figure 4**, A and B).

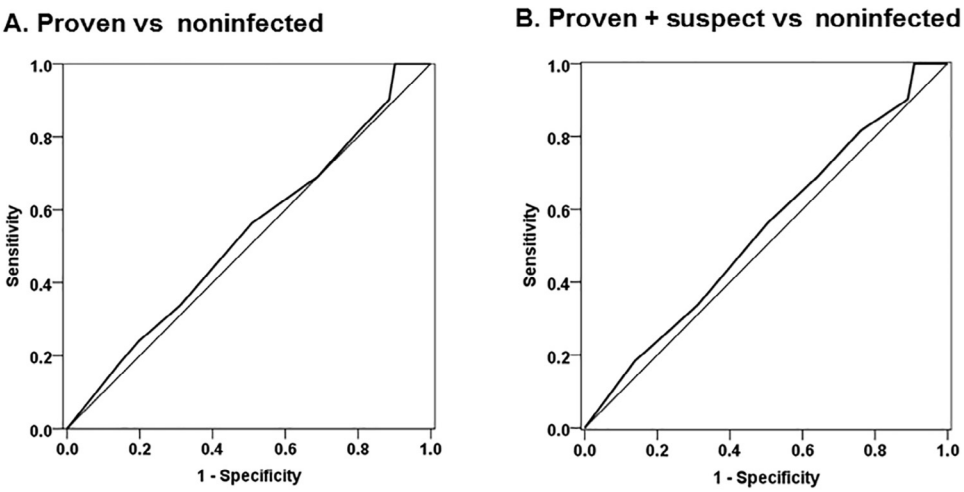

Figure 4. ROC for serial neutrophil values using the neutrophil value score to predict the absence of LOS for **A,** proven and **B,** proven + suspect vs noninfected. The *diagonal line* represents the line of identity where ties are encountered.

Discussion

Although the incidence of neonatal LOS has decreased, many infants continue to be evaluated for LOS and receive prolonged antimicrobial therapy because of nonspecific clinical signs of LOS and the fear of long-term adverse outcomes.[2-6] This is particularly true in infants with a central venous catheter.[3,6,7] Clinicians, therefore, have sought screening tools or biomarkers to identify infants who should be treated or, in the absence of positive blood cultures, have antibiotics stopped at 36-48 hours to decrease exposure and adverse effects. The CBC, in particular the neutrophil values derived from the differential cell count, has been used extensively.[2,10,13] However, the positive and negative predictive values remain unclear because of variation in the reference or cutoff values that have been used. We used the Manroe RR beyond 72 hours of age to determine whether the neutrophil values obtained in infants with central venous catheters accurately predicted the presence or absence of LOS. We observed that a single CBC obtained at the time of initial LOS evaluation, ie, a "screening" CBC, had poor positive and negative predictive values when an established neutrophil RR was used. Moreover, the addition of serial neutrophil values obtained at 12 and 24 hours and their assessment alone or as the neutrophil value score also had poor prediction for the presence or absence of LOS using these RR.

Biomarkers are sought for nearly every disease state to improve identification of patients either with or without the disease of interest.[14,15] Most often, they are used to identify the presence of a disease to selectively treat the population at greatest risk and provide a low risk-benefit ratio. When carefully assessed, the majority of biomarkers have relatively poor prediction, often due to the RR or cutoff values chosen or the lack of validation.[15,16] To validate a biomarker, RR should ideally be constructed from patients with and without the disease of interest to identify benign events or conditions that result in values outside the normal range.[16,17] This is often difficult because "well" patients with benign conditions must be screened. Manroe et al[11] established the first neonatal neutrophil RR extending from birth to 30 postnatal days. Because of the broad differences in physiologic factors, risks at birth and potential for change over time after birth, numerous benign and noninfectious factors were examined to determine whether the absolute neutrophil values accurately identified EOS. Furthermore, the values were obtained from carefully validated manual differential cell counts provided by the hospital laboratory. As anticipated, several noninfectious clinical conditions significantly altered the distribution of neutrophil values after birth, including maternal hypertension and fever, perinatal asphyxia, hemolytic disease, and preterm birth, to name a few.[11] Moreover, the upper and lower limits of the RR changed significantly during the first 96 postnatal hours; thus, single cutoff values were inappropriate.

Taking this into account, especially the change over time, the neutrophil values had a positive predictive value of 0.84 for EOS; moreover, the negative predictive value was >0.9.[10] These findings were confirmed by Rodwell et al.[18] Subsequent investigators confirmed the time-dependent changes in the ATN RR, but few examined the ATI or I:T.[19,20] Mikhael et al[12] recently validated the Manroe RR in the first 96 postnatal hours, confirmed the high negative predictive value for EOS, and introduced the use of serial neutrophil values in the neutrophil value score, which had a negative prediction >0.85 for the absence of EOS. This has not been examined as a tool to predict LOS, and in most cases, arbitrary values have been used to indicate normal cutoffs.[13]

To address these issues, we examined the Manroe RR ≥72 postnatal hours, which are considerably more static with minimal change from 72 to 96 hours and no change between 96 hours and 30 days postnatal, during which time the range for the ATN RR is 1800 to 5400 cells/mm^3, ATI 0-650 cells/mm^3, and I:T ≤0.12.[11] Because these ranges are unchanged after 96 postnatal hours, it is likely they are valid for the group of infants evaluated >30 postnatal days. Only 3% of noninfected episodes exhibited neutropenia, confirming the lower cutoff for ATN, which is important as neutropenia often is considered a sign of sepsis. However, neutrophilia occurred in >30%, extending recent observations in the first 96 hours after birth.[12,20,21] Although it is unlikely this is due to high altitude, as originally assumed, there presently is no explanation. It could reflect an unrecognized benign condition, eg, excessive catecholamine release associated with apnea or feeding intolerance that if increased might also trigger a sepsis evaluation. This should be examined in future studies.

An elevated I:T, or shift to the left, also is used frequently to identify neonatal infection. Although an I:T >0.12 occurred in only 17% of noninfected episodes, this exceeded the expected 10% based on the Manroe's RRs. Importantly, there were no differences in the distribution of neutrophilic values in the proven, suspect, and noninfected episodes, demonstrating substantial overlap and no clear separation. Thus, the noninfected values cannot be used to assess the original RR. It also should be noted that the original and present neutrophilic values were generated from manual differential cell counts. Although Christensen et al reported a similar pattern in ATN values generated by automated differential neutrophil counts beyond 72 postnatal hours, the automated ATN counts included band neutrophils and the ATI included only promyelocytes and younger neutrophils.[20] This differs from the Manroe definitions and thus, the RRs cannot be used to determine normal vs abnormal neutrophilic values generated by automated cell counters. Further studies are needed to demonstrate the utility of these RR for neutrophilic values generated by manual differentials from birth to 30 days. Our hospital laboratories continue to generate manual differentials for all neonates as standard of care. The use of neutrophil values generated by automated CBC and differential remain to be validated for LOS.

A "screening CBC" is frequently used to assess the risk for LOS but again in the absence of any validation.[2] We and others have recently shown the lack of efficacy in using a "screening" CBC to detect the risk for EOS[12,22]; this has not been examined for LOS. We now report that as in EOS, there is no

valid use of a "screening" CBC in the evaluation for LOS, even in the presence of a central venous catheter. This is not only true for the occurrence of LOS as a whole, but also for all gram-positive organisms, which, as observed in this study, account for ≥70% of LOS infections.[1,3,6,7] An exception is the initial I:T value obtained in the presence of gram-negative bacteria where the AUC for the first CBC was 0.84; however, this was not observed at 12 and 24 hours. Because >70% of infecting organisms in LOS are gram positive, this practice is not cost effective. Unlike EOS, serial CBCs with manual neutrophil counts also did not accurately detect the presence or absence of LOS irrespective of the infecting organism.[12] This is also true for the neutrophil value score, which has high specificity for EOS and is an excellent tool for stopping antibiotic therapy at 24-36 hours after their start.[12]

There were no cases of meningitis or fungal infection over the 3 years of this study, even though every infant had a spinal fluid culture. Further, there were 9 instances in which the urine culture was positive in the proven and suspect groups, ie, 8% of infants treated. It is likely the 4 suspect infants with blood culture–negative infection were treated for the positive urine cultures. Finally, the duration of antibiotics was significantly shorter in the noninfected group but was still on average approximately 3 days. To shorten this to 48 hours, we have changed the antibiotic order set to include a hard stop at 48 hours that prevents excessive exposure.[23]

The study may be considered limited by the inclusion of neonates and infants in a single NICU, and some would argue the inclusion of only infants with a central venous catheter is restrictive. This population was chosen because of their increased risk for LOS and the likelihood we would have sufficient patients in all 3 arms of the study with a complete evaluation because central venous catheters are commonly used in the NICU to provide supplemental nutrition to neonates born preterm.[6,7] It would be important to see similar studies performed in other institutions and to include other infants at risk of LOS. Another limitation might be the use of manual differential counts; however, as noted earlier, we have found this to be accurate and highly effective for examining EOS in our hospitals and NICUs. Nonetheless, the use of automated CBCs should be examined and further validated as it may prove cost effective.

In conclusion, the original RRs for ATN, ATI, and I:T reported by Manroe et al[10,11] do not aid in accurately predicting the presence or absence of LOS and do not separate noninfected episodes from proven or proven + suspect episodes between birth and >30 postnatal days. Moreover, the initial CBC or "screening" CBC as well as serial CBCs and the neutrophil value score are poor predictors for LOS. Although the neutrophilic RRs appear to extend beyond 30 days of life, based on data from infants >4 months of age, this requires additional study before implementation.[24] The neutrophilia originally seen in the first 72-96 hours after birth actually extends to 30 postnatal days and its etiology remains unclear and in need of further study; if it is benign, it might be corrected for in future studies. If the CBC and neutrophilic values are poor predictors of LOS, other biomarkers should be pursued, adequately identified, and validated after defining their normal distribution corrected for important developmental and birth-related variables.[6,25,26] Until then, if LOS is suspected, we continue to recommend that treatment be quickly initiated because of the high risk associated with LOS and the duration of antibiotics be based on culture results and the clinical status. ■

Submitted for publication Apr 20, 2018; last revision received Aug 15, 2018; accepted Sep 10, 2018

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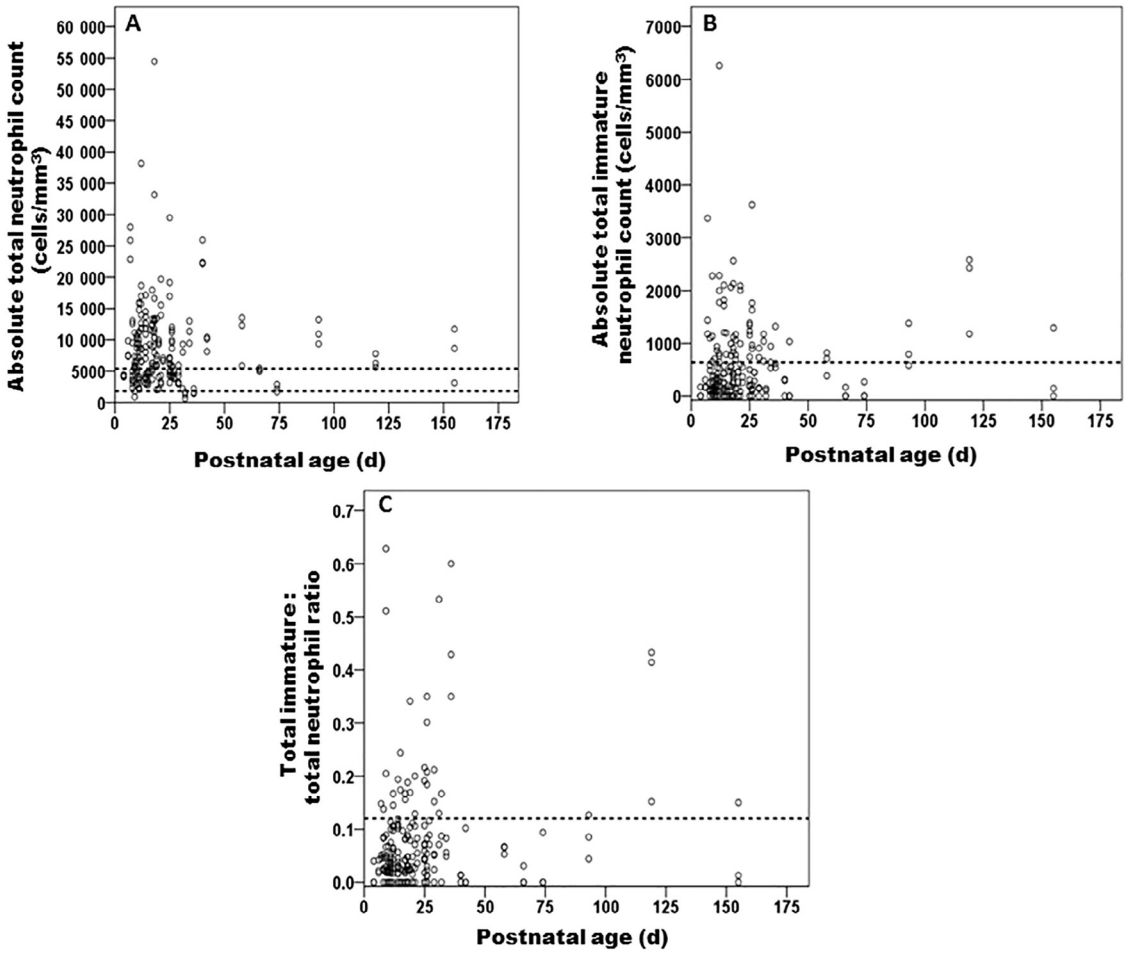

Figure 1. Neutrophil RRs from birth to 175 days after birth derived from Manroe et al[11] for the **A,** ATN and **B,** ATI and **C,** I:T proportion. Values from 213 CBCs are illustrated. The cutoff values for each cell count are represented by a *dotted line*; only ATN (**A**) has an upper and lower cutoff.

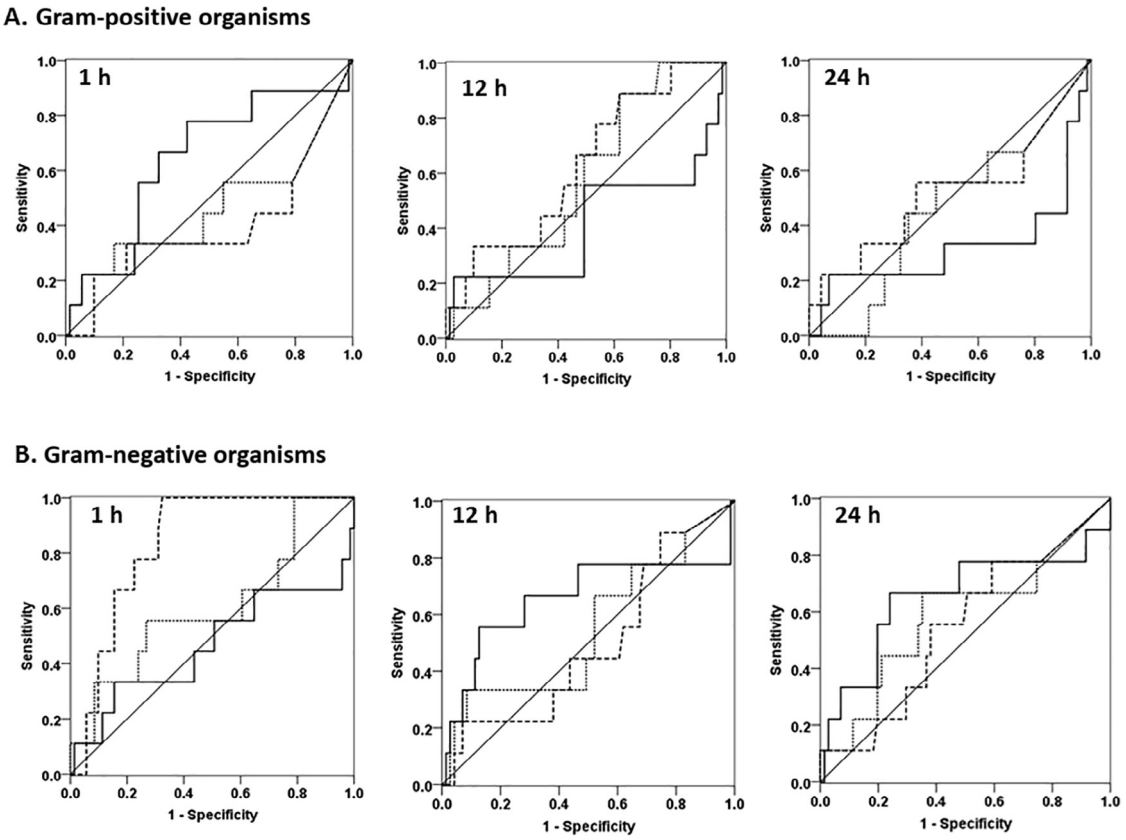

Figure 3. ROC curves for ATN (*solid lines*), ATI (*dotted lines*), and the I:T (*dashed lines*) at the time of initiating an evaluation for LOS and at 12 and 24 hours later comparing proven vs noninfected for **A,** gram-positive and **B,** gram-negative organisms. The *diagonal line* represents the line of identity where ties are encountered.