



## Letter to the Editor

### Elevated mean neutrophil volume+CRP is a highly sensitive and specific predictor of neonatal sepsis

Sir,

Neonatal sepsis comprises a major problem in India with septicemia and pneumonia accounting for 52% of deaths in home-cared rural-born neonates [1]. Early detection is challenging as clinical signs are subjective, and the gold standard, a positive blood culture, requires 1–3 days [1, 2]. Cases of suspected but culture-negative sepsis outnumber culture-positive ones by at least 15–20 times [1]. Inexpensive diagnostic alternatives like complete blood counts, neutrophilia, immature neutrophils, and the immature-to-total neutrophil (IT) ratio are relatively insensitive [1–3]. Other markers like C-reactive protein (CRP), interleukin (IL)-6, procalcitonin, calprotectin, lipopolysaccharide-binding protein, and neutrophil CD64 expression as well as novel molecular/proteomic techniques have been investigated [1, 2, 4].

Automated hematology analyzers yield several electronic and optical leukocyte indices like volumes of leukocyte subsets, cellular electrical conductivity, optical light scatter, etc. These have shown promise in early and sensitive diagnosis of diseases characterized by leukocytic abnormalities [3, 5–9]. The leukocyte differential in Beckman Coulter analyzers (Beckman Coulter, Miami, FL, USA) is based on VCS technology (volume, conductivity, and scatter) that quantifies leukocyte cell volume by voltage impedance (V); cytoplasmic and nuclear density by radio frequency conductivity (C); and cytoplasmic granularity and nuclear complexity by laser light scatter (S) [3]. VCS data are obtained simultaneously with the differential count without any requirement of additional testing, extra sample, reagents, time, or expenditure. We evaluated its performance as a potential marker of neonatal sepsis.

The study setting was a busy referral neonatal intensive care unit (NICU) in a tertiary-care teaching hospital. After institutional Ethics Committee clearance, venous blood samples were drawn from 94 neonates aged 0–28 days admitted consecutively to the NICU in whom sepsis was suspected along with 36 gestation-matched healthy controls with no clinical or laboratory suspicion

of infection. Complete blood counts and VCS parameters were obtained from Beckman Coulter™ LH750 or LH755 instruments (Beckman Coulter). Romanowsky-stained blood films were examined, and leukocyte differential count, neutrophil percentage, immature neutrophil percentages (i.e., bands, metamyelocytes, myelocytes, and promyelocytes), and IT ratio were calculated. Simultaneous blood cultures and CRP measurement (Beckman Synchron LX-20 Pro; Beckman Coulter) were sent in all babies. Urine, CSF, and/or other site cultures were performed as necessary.

All neonates were followed up till discharge, death, or leave against medical advice. They were subsequently divided into three groups by the clinical investigator (a senior experienced neonatologist) blinded to the VCS results: (i) definite sepsis (compatible clinical background and at least one culture report positive within 48–72 h,  $n = 35$ ), (ii) probable sepsis (all cultures negative but clinical or other laboratory evidence necessitating institution of antibiotic therapy for probable infection,  $n = 28$ ), and (iii) no sepsis (transient signs and symptoms subsequently resolving without antibiotics,  $n = 31$ ). Clinical suspicion in the 'probable sepsis' group was based on indicators including hypothermia or fever, lethargy, poor cry, poor feeding, poor perfusion, hypotonia or absent neonatal reflexes, bradycardia or tachycardia, respiratory distress, hypoglycemia or hyperglycemia, metabolic acidosis, physician-documented seizures, abdominal distension, vomiting, diarrhea, or jaundice. These infants were started on presumptive antimicrobial therapy despite the negative culture report(s).

For statistical analysis, the first two groups (definite and probable sepsis,  $n = 63$ ) were considered as 'sepsis', and the last group (no sepsis) was combined with the healthy controls as 'negative-for-sepsis' ( $n = 67$ ). Descriptive statistics as well as receiver-operating-characteristic (ROC) curves with area-under-curve (AUC) analysis were used to calculate and compare the diagnostic efficiencies of VCS vs. conventional parameters as well as that of various combinations of parameters (SPSS for Windows, version 16.0; IBM Corp, Armonk, NY, USA). In addition, the software 'EMMA' (Effective modelling of

molecular activity)' [Sukhachev DV, Zefirov NS. 10th European Symposium on Structure-Activity relationships: QSAR and molecular modeling. Barcelona, 1994, p. A104] was used to generate 'best' regression equations from combinatorial algorithms of different selected parameters. This software has been previously validated by another group for its utility in analyzing multiple parameters in neonatal sepsis [8]. The aim was to ultimately generate equations that can be incorporated into the LIS or AUTOANALYZER software to generate report flags for neonatal sepsis.

Of the 94 study group patients, 61 were referrals from other centers. All 36 healthy control babies were delivered in our hospital. Of the 35 culture-positive cases, *Klebsiella pneumoniae* and *Enterobacter spp.* were the commonest isolates (six patients each). Less common agents included *Escherichia coli*, various Gram-positive cocci, *Acinetobacter spp.*, *Burkholderia cepacia*, and four patients with Candidal sepsis.

Among all indices studied, hemoglobin, platelet count, absolute lymphocyte count, and mean neutrophil scatter (MNS) and conductivity (MNC) were significantly lower in sepsis group while total leukocyte count, absolute neutrophil count, absolute monocyte count, mean neutrophil volume (MNV), and CRP were significantly higher in the sepsis group as compared to negative-for-sepsis group (Table 1). On ROC-curve analysis, hemoglobin, MNV, and CRP were most discriminatory between groups (highest AUC), and these parameters at the ROC-curve determined cutoffs (MNV > 154.2 arbitrary units, CRP > 7.0) were included in combinatorial regression functions (using EMMA™).

Table 2 gives the performance characteristics of the salient parameters using cutoff values derived from ROC-curve analysis. MNV > 154.2 had 95.5% sensitivity and 82.1% specificity (AUC 0.93) for neonatal sepsis followed by CRP > 7.0 mg/L with 78.9% sensitivity and 96.3% specificity (AUC 0.89). Most significantly, the combination

**Table 1.** A comparison of clinical and laboratory parameters and neutrophil VCS indices in the sepsis versus negative-for-sepsis groups

|  | Sepsis group<br>(n = 63) | Negative for sepsis<br>group (n = 67) | P-value<br>(unpaired t-test) |
|--|--------------------------|---------------------------------------|------------------------------|
| <b>Clinical parameters</b>                                       |                          |                                       |                              |
| Age, mean ± SD (days)  | 11 ± 9                   | 16 ± 5                                | –                            |
| Males : Females  | 47 : 16                  | 39 : 28                               | –                            |
| Proportion born outside study hospital (%)                       | 92                       | 0                                     | –                            |
| Gestation period, mean ± SD (weeks)                              | 32.1 ± 4.1 (27–40)       | 34.3 ± 4.6 (27–41)                    | –                            |
| Mortality (n, %)   | 8*, 12.7%                | 1†, 1.5%                              | –                            |
| <b>Laboratory parameters (all values expressed as mean ± SD)</b> |                          |                                       |                              |
| Hemoglobin (gm/dL)   | 11.9 ± 4.1               | 15.1 ± 3.2                            | <0.0001                      |
| Total leukocyte count (×10 <sup>9</sup> /L)                      | 23.5 ± 12.8              | 16.5 ± 10.3                           | 0.0008                       |
| Platelet count (×10 <sup>9</sup> /L)                             | 82 ± 41                  | 119 ± 39                              | <0.0001                      |
| Absolute neutrophil count (×10 <sup>9</sup> /L)                  | 19.9 ± 8.1               | 10.6 ± 6.2                            | <0.0001                      |
| Absolute lymphocyte count (×10 <sup>9</sup> /L)                  | 3.4 ± 2.8                | 4.9 ± 2.1                             | 0.0007                       |
| Absolute monocyte count (×10 <sup>9</sup> /L)                    | 1.7 ± 0.7                | 0.8 ± 0.5                             | <0.0001                      |
| Absolute eosinophil count (×10 <sup>9</sup> /L)                  | 0.5 ± 0.7                | 0.4 ± 0.5                             | 0.3482                       |
| Band cell%   | 4.0 ± 5.6                | 3.3 ± 3.3                             | 0.3835                       |
| Metamyelocyte%   | 0.6 ± 1.1                | 0.3 ± 0.6                             | 0.0539                       |
| Myelocyte%   | 1.2 ± 1.6                | 0.9 ± 1.3                             | 0.2416                       |
| IT ratio   | 0.12 ± 0.1               | 0.10 ± 0.1                            | 0.2566                       |
| C-reactive protein, mean ± SD (mg/L)                             | 68.8 ± 74.2              | 2.6 ± 11.0                            | <0.0001                      |
| <b>Neutrophil VCS indices</b>                                    |                          |                                       |                              |
| Mean neutrophil volume (MNV)                                     | 162.2 ± 19.2             | 148.9 ± 7.8                           | <0.0001                      |
| MNV standard deviation   | 36.4 ± 8.9               | 33.6 ± 5.8                            | 0.0345                       |
| Mean neutrophil conductivity (MNC)                               | 141.3 ± 5.6              | 143.7 ± 5.6                           | 0.0160                       |
| MNC standard deviation   | 11.3 ± 3.4               | 10.5 ± 2.7                            | 0.1386                       |
| Mean neutrophil scatter (MNS)                                    | 143.7 ± 13.0             | 148.1 ± 11.4                          | 0.0419                       |
| MNS standard deviation   | 15.7 ± 3.3               | 15.7 ± 4.1                            | 1.0000                       |

\*Outcomes were unknown in two sepsis group babies taken away against medical advice.

†Mortality was nil in healthy controls (n = 36).

**Table 2.** Performance characteristics of salient parameters studied. Blood culture positivity and/or clinical/laboratory features necessitating antibiotic therapy were taken as the gold standard for sepsis

|             | IT ratio >0.09 | CRP >7 mg/L | IT ratio >0.09 +<br>CRP >7 mg/L | MNV >154.2 | MNV >154.8 +<br>CRP >7 mg/L |
|-------------|----------------|-------------|---------------------------------|------------|-----------------------------|
| Sensitivity | 66.7           | 78.9        | 84.2%                           | 95.5       | 100.0                       |
| Specificity | 67.9           | 96.3        | 92.9%                           | 82.1       | 85.7                        |
| AUC         | 0.63           | 0.89        | 0.91                            | 0.93       | 0.97                        |

IT ratio, Immature to total neutrophil ratio; CRP, C-reactive protein; MNV, Mean neutrophil volume.

of MNV >154.8 and CRP >7.0 mg/L had 100% sensitivity and 85.7% specificity (AUC 0.97) for neonatal sepsis. In comparison, IT ratio >0.1 + CRP > 7.0 showed sensitivity of 84.2% and specificity of 92.9% (AUC 0.91). The function designed by regression analysis [sepsis if  $-1.512$  to  $0.05 \times (\text{Hemoglobin}) + 0.0166 \times (\text{MNV}) + 0.0022 \times (\text{CRP})$  is  $>0.4628$ ] showed 94.1% sensitivity and 100% specificity with AUC 0.98. This function is suitable for incorporation into laboratory information systems with access to biochemistry and hematology analyzers for the generation of a 'sepsis flag'.

Of the morphological parameters, immature neutrophils (percentage bands + metamyelocytes + myelocytes + promyelocytes) alone had a high specificity of 89.3% but unacceptably low sensitivity of 27.3% (AUC 0.55). IT ratio >0.09 performed modestly with 66.7% sensitivity and 67.9% specificity with AUC 0.634. Alternative within-group statistical analyses (for instance, comparing the culture-positive sepsis patients with culture-negative ones or comparing healthy controls with no-sepsis babies) yielded lower numbers of significantly different parameters, but the analyses were underpowered due to the fewer number of patients. These diagnostic distinctions were also considered clinically and therapeutically less significant with respect to the urgency of initiating antibiotic therapy.

A few studies have previously analyzed cell population data to diagnose neonatal sepsis. Our results are similar, but not identical to those recently reported by Celik *et al.* [5, 8] who also used similar statistical modeling. Although they too found significantly higher MNV in septic neonates, unlike us they reported significant differences in the mean neutrophil conductivity and distribution width. A combination of IL-6, CRP, and MNV achieved highest sensitivity and specificity in their study (94 and 88%, respectively) [5]. The subtle discrepancies between the findings may be due to differences in patient populations (mean ages of study groups 5–11.5 days [5] as compared to 11–16 days in our study) or as pointed out by others, may illustrate the need for interlaboratory standardization of VCS indices [6]. Current quality control for VCS indices is based on testing with specific control material.

Raimondi *et al.* [6] had previously recommended VCS indices as a screening test for late-onset sepsis in very low birth infants. MNV > 148 + CRP >9 mg/L had shown 95% sensitivity and 97% specificity (AUC 0.96). Our study which also looked at relatively later-onset sepsis (mean ages 11 and 16 days, Table 1) corroborates their findings.

Morphological parameters like immature neutrophils and IT ratio were readily outperformed by VCS indices in our study. This could be because VCS analysis quantifies parameters imperceptible to the human observer like cell volume, light transmission and electrical conductivity, imparting objectivity, and reproducibility. They are also less subject to sampling variation, because unlike a 100 or 200-cell differential, the VCS indices are derived from up to 8000 cellular events. Similar effective concepts are also under development by other manufacturers. Zimmerman *et al.* [9] recently suggested the Granularity Index of the SYSMEX XE-5000 hematology analyzer (Sysmex Corp, Kobe, Japan) as a replacement for manual microscopy of neutrophils with toxic granulation in inflammatory diseases.

We compared babies with definite and probable infection with a combined group of healthy as well as non-septic neonates. This is because the 'gold standard', a positive blood culture, was negative (i.e., insensitive) in neonates who otherwise had a high likelihood of infection on evaluation by an experienced neonatologist. Cultures may return negative for several reasons: prior antibiotic therapy (often started by the primary care pediatrician while referring sick neonates), small-volume blood samples lowering yield, or fastidious microorganisms requiring special growth conditions [1, 10], thus underscoring a need for new diagnostic modalities.

The 'no sepsis' group results suggest that VCS data together with CRP distinguish septic neonates from both normals and from disease controls. More evidence needs to be accrued to demonstrate if VCS can distinguish neonates with acute infections from those with noninfectious stress responses, hemorrhage, other hematologic disorders, immunosuppression, viral infections, etc. Although we did not follow up for resolution of VCS

indices beyond discharge, it has been demonstrated that successful treatment may normalize indices [5, 8].

In conclusion, the MNV + CRP criterion is a potentially useful tool for the evaluation of neonates with proven as well as suspected sepsis. Its use along with other VCS parameters in regression equations that are calculable by hospital information systems could improve timely recognition of serious infections in this vulnerable population.

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## Prior presentation

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## References

1. Kler N, Garg P, Saluja S. Neonatal infections. In: Recent Advances in Pediatrics, Volume 8. Gupte S (ed.). New Delhi: Jaypee Brothers Publishers; 1998: 373–400.
2. Srinivasan L, Harris MC. New technologies for the rapid diagnosis of neonatal sepsis. *Curr Opin Pediatr* 2012;24:165–71.
3. Bain BJ. *Blood Cells: A Practical Guide*, 4th ed. Oxford, UK: Blackwell; 2006: 20–60.
4. Benitz WE. Adjunct laboratory tests in the diagnosis of early-onset neonatal sepsis. *Clin Perinatol* 2010;37:421–38.
5. Celik IH, Demirel G, Aksoy HT, Erdevi O, Tuncer E, Biyikli Z, Dilmen U. Automated determination of neutrophil VCS parameters in diagnosis and treatment efficacy of neonatal sepsis. *Pediatr Res* 2012;71:121–5.
6. Raimondi F, Ferrara T, Capasso L, Sellitto M, Landolfo F, Romano A, Grimaldi E, Scopacasa F. Automated determination of neutrophil volume as screening test for late-onset sepsis in very low birth infants. *Pediatr Infect Dis J* 2010;29:288.
7. Mardi D, Fwity B, Lobmann R, Ambrosch A. Mean cell volume of neutrophils and monocytes compared with C-reactive protein, interleukin-6 and white blood cell count for prediction of sepsis and nonsystemic bacterial infections. *Int J Lab Hematol* 2010;32:410–18.
8. Celik IH, Demirel G, Sukhachev D, Erdevi O, Dilmen U. Neutrophil volume, conductivity and scatter parameters with effective modeling of molecular activity statistical program gives better results in neonatal sepsis. *Int J Lab Hematol* 2013;35:82–7.
9. Zimmermann M, Cremer M, Hoffmann C, Weimann K, Weimann A. Granularity Index of the SYSMEX XE-5000 hematology analyzer as a replacement for manual microscopy of toxic granulation neutrophils in patients with inflammatory diseases. *Clin Chem Lab Med* 2011;49:1193–8.
10. Maki DG. Microbiologic diagnosis of blood culture-negative sepsis by hemofiltration. *Crit Care Med* 2004;32:1075–7.