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REVIEW ARTICLE

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Diagnosis of neonatal sepsis: the past, present and future

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ABSTRACT: Sepsis remains a significant cause of neonatal mortality and morbidity, especially in low- and middle-income countries. Neonatal sepsis presents with nonspecific signs and symptoms that necessitate tests to confirm the diagnosis. Early and accurate diagnosis of infection will improve clinical outcomes and decrease the overuse of antibiotics. Current diagnostic methods rely on conventional culture methods, which is time-consuming, and may delay critical therapeutic decisions. Nonculture-based techniques including molecular methods and mass spectrometry may overcome some of the limitations seen with culture-based techniques. Biomarkers including hematological indices, cell adhesion molecules, interleukins, and acute-phase reactants have been used for the diagnosis of neonatal sepsis. In this review, we examine past and current microbiological techniques, hematological indices, and inflammatory biomarkers that may aid sepsis diagnosis. The search for an ideal biomarker that has adequate diagnostic accuracy early in sepsis is still ongoing. We discuss promising strategies for the future that are being developed and tested that may help us diagnose sepsis early and improve clinical outcomes.

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IMPACT:

- Reviews the clinical relevance of currently available diagnostic tests for sepsis.
- Summarizes the diagnostic accuracy of novel biomarkers for neonatal sepsis.
- Outlines future strategies including the use of omics technology, personalized medicine, and point of care tests.

INTRODUCTION

Neonatal sepsis is a clinical syndrome characterized by nonspecific signs and symptoms caused by invasion by pathogens.[1,2] Sepsis is deemed culture-proven if confirmed by microbial growth on blood cultures or other sterile bodily fluids. Debate exists over the occurrence of culture-negative sepsis and whether antibiotics should be continued in culture-negative cases.[3] Sepsis is categorized as early onset if diagnosed within the first 72 h of life, which is due to perinatal risk factors, or late-onset if diagnosed after 72 h and secondary to nosocomial risk factors. Neonatal sepsis is still a major cause of morbidity and mortality despite advances in neonatal medicine.[4] Incidence varies from 1 to 4 cases per 1000 live births in high-income countries, but as high as 49–170 cases in low- and middle-income countries with a case fatality rate up to 24%.[5–8] Survivors of neonatal sepsis are at increased risk for adverse neurodevelopmental outcomes including cerebral palsy, hearing loss, visual impairment, and cognitive delays even in those whose cultures were negative but were treated with antibiotics.[9,10]

The diagnosis of confirmed sepsis relies on conventional microbiologic culture techniques, which can be time-consuming.[11] Despite the high sensitivity in detecting low bacterial loads (1–4 colony-forming unit (CFU)/mL), many providers view negative blood cultures with skepticism when presented with a sick infant.[12] The diagnosis "culture-negative" sepsis or "clinical sepsis" has led to a 10-fold increase in antibiotic use in neonates with evidence of unintended harm including increased risk for necrotizing enterocolitis, fungal infections, bronchopulmonary dysplasia, and death.[12]

Advances in rapid culture techniques, antibiotic stewardship, and bundled approaches to prevent central line-associated bloodstream infections have reduced morbidity and mortality from neonatal sepsis.[13,14] Newer molecular approaches and nonculture-based methods to assist in timely detection and accurate diagnosis of sepsis are needed. Current biomarkers and adjunct hematological indices used in routine clinical practice have limited value and are difficult to interpret due to low sensitivity and changing normal ranges during the neonatal period.[15,16] An ideal marker should have sensitivity and negative predictive value (NPV) approaching 100%; specificity and positive predictive value (PPV) over 85%.[17,18] None of the biomarkers or combination of biomarkers have adequate diagnostic accuracy to be used reliably in the diagnosis of neonatal sepsis.[19] We aim to review the past and current diagnostic modalities and present some insight on future diagnostic strategies in neonatal sepsis (Fig. 1).

PATHOPHYSIOLOGY OF NEONATAL SEPSIS

Host immune responses including cytokines and chemokines during neonatal sepsis may aid in the diagnosis and/or assessing the severity of sepsis. A summary of the biomarkers associated with host immune pathways that change during sepsis is depicted in Fig. 2. Paneth cells and intestinal lymphoid cells produce

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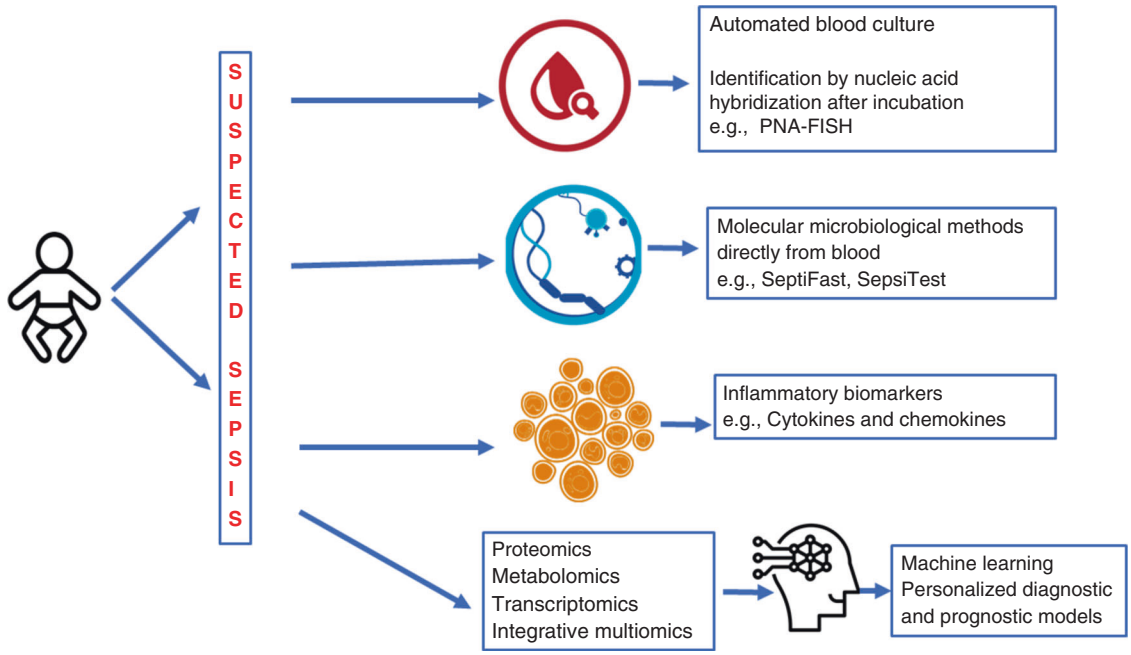

Fig. 1 A Schematic on the categories of diagnostic tests available for neonatal sepsis. Traditional methods of blood cultures have changed to automated blood culture monitoring for bacterial growth by CO_2 detection. Newer tests involve rapidly identifying organisms from positive cultures by fluorescent in situ hybridization techniques. Molecular microbiological diagnostics using PCR for bacterial and fungal genes can be applied directly to blood specimens. Inflammatory biomarkers including CRP, procalcitonin, and cytokines are another category of adjunctive diagnostic tests. Multiomic technology enables us to scour genome-wide gene expression, protein and metabolites for developing diagnostic tests and prognostic models.

interleukin-17 (IL-17), which has a role in local defense and development of systemic inflammatory response syndrome.[20] Respiratory epithelia secrete antimicrobial proteins and peptides including cathelicidin and β-defensins.[21] Gram-positive microorganisms and their cell wall lipoteichoic acid signal through toll-like receptor-2 (TLR-2), while gram-negative microorganisms and their secreted lipopolysaccharide (LPS) signal through TLR-4 receptors.[22] These signaling cascades are associated with the production of nuclear factor-κB-dependent inflammatory cytokines and chemokines. Nucleotide-binding and oligomerization domain-like receptors lead to the production of IL-1β and IL-18 by a protein complex called the inflammasome.[23] Activation of pathogen recognition receptors results in the generation of inflammatory mediators such as IL-1β, IL-6, IL-8, IL-12, IL-18, interferon-γ (INF-γ) and tumor necrosis factor-α (TNF-α).[24] Proinflammatory cytokines activate endothelial cells leading to increased expression of cell adhesion molecules such as soluble intercellular adhesion molecules, selectins, angiopoietins, CD11b, and CD18.[25] Chemokines including CXCL10, CCL5 (RANTES), and CCL3, and complement proteins such as C3a and C5a cathelicidin and defensins are also stimulated by proinflammatory cytokines.[26] Damage-associated molecular patterns (DAMPs, alarmins), such as high-mobility group box-1 and uric acid, are released from damaged cells and induce cytokine production, coagulation cascade, and regulate polymorphonuclear cell function.[27] Anti-inflammatory cytokines such as transforming growth factor-β, IL-4, IL-10, IL-11, and IL-13 are expressed to control and balance inflammation.[28] Acute-phase reactants (APRs) such as C-reactive protein (CRP), procalcitonin (PCT), serum amyloid A (SAA) are produced predominantly in the liver in response to complement activation, pathogen-associated molecular patterns (PAMPs) activity, and proinflammatory cytokine secretion.

CURRENT METHODS TO DIAGNOSE NEONATAL SEPSIS

Microbiological culture methods

Conventional culture techniques remain the "gold standard" to confirm the diagnosis of neonatal sepsis. The introduction of automated systems that detect the presence of growth from bacterial CO_2 production has reduced the time to organism detection to 24–48 h.[29,30] Factors that may influence the recovery of pathogens from the blood include amount of blood volume obtained, timing of collection, and number of samples collected.

In neonates, the presence of low or intermittent bacteremia and maternal intrapartum antimicrobial exposure may decrease the sensitivity of blood cultures.[12,31] The delay in pathogen identification and antibiotic susceptibility testing increases exposure to broad-spectrum antibiotics, which may lead to bacterial antibiotic resistance and delay in targeted antimicrobial therapy.[9,32,33] The volume of blood sampled for cultures is the single most important factor influencing the recovery of pathogens from blood cultures.[34] However, collection of optimal blood volume can be difficult in extremely preterm infants and repeated phlebotomy may increase the risk of requiring blood transfusions. Schelonka et al. reported that a blood culture volume of 1 mL injected into pediatric blood culture bottles had excellent sensitivity even if organisms were present at very low concentrations (<4 colony-forming units (CFU)/mL).[31]

The need for obtaining anaerobic cultures in neonates before commencing antibiotics is unclear.[35] The overall incidence of clinically significant anaerobic isolates found in a neonatal population was 0.2% of all blood cultures performed.[36] Previous studies showed that the use of anaerobic blood cultures led to increased identification of both aerobic and facultative anaerobic bacteria.[37] Créixems et al. reported that among 10,024 paired blood cultures (aerobic and anaerobic), 19% of patients with bacteremia would have been missed if aerobic cultures alone were used, not including the three strictly anaerobic infections identified.[38] In

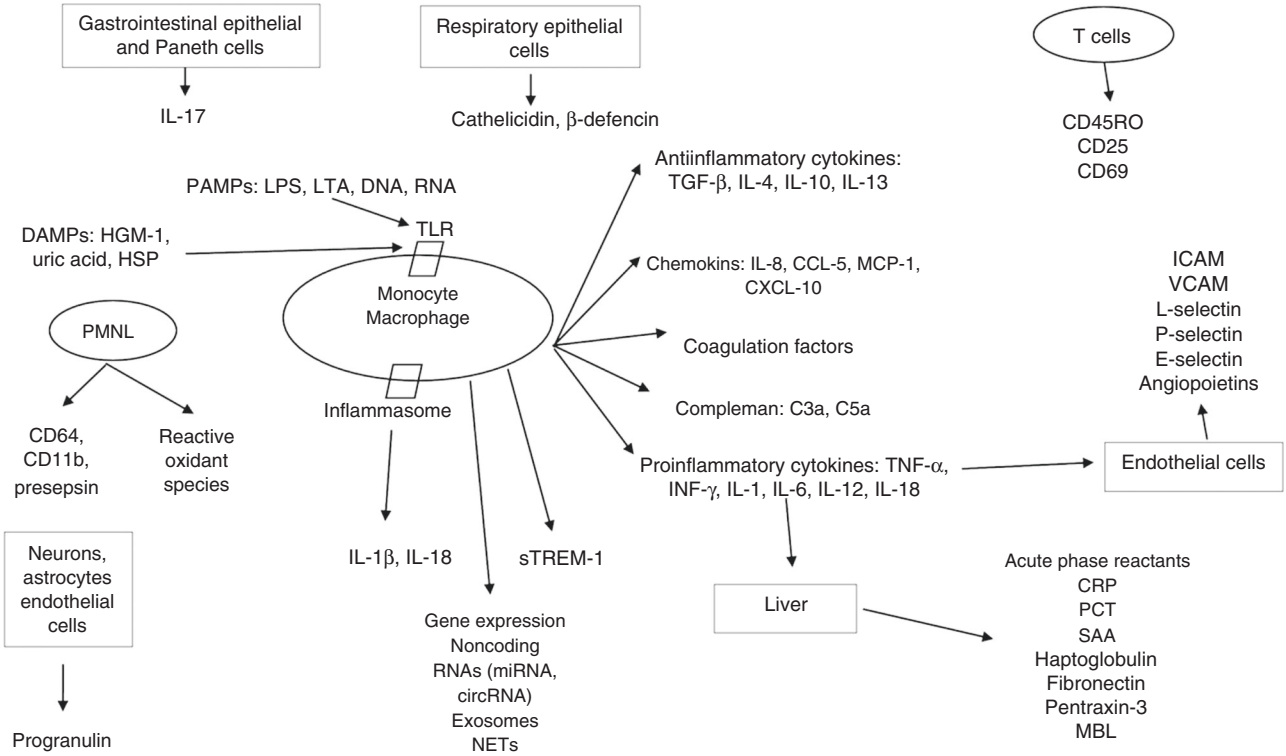

Fig. 2 The relationship between host immunity and biomarkers. CD cluster of differentiation, sTREM-1 soluble triggering receptor expressed on myeloid cells-1, ICAM intracellular adhesion molecule, VCAM vascular cell adhesion molecule, RNA ribonucleic acid, DNA deoxyribonucleic acid, DAMPs damage-associated molecular patterns, HGM-1 high-mobility group box 1, LPS lipopolysaccharide, LTA lipoteichoic acid, NETs neutrophil extracellular traps, TLR toll-like receptor, HSP heat-shock protein, TNF-α tumor necrosis factor-α, INF-γ interferon-γ, IL interleukin, MCP-1 monocyte chemoattractant protein-1, CXCL-10 chemokine ligand-10.

contrast, Dunne et al. found increased sensitivity in isolating aerobic and facultative anaerobic isolates from pediatric patients when two aerobic blood cultures were performed versus paired aerobic/anaerobic cultures.[39] It is unclear whether treating anaerobes in routine sepsis management in neonates improves clinical outcomes.

Rapid testing methods from positive blood cultures

Several diagnostic systems have been developed for the rapid identification of organisms found in positive blood cultures and provide faster turnaround times when compared to conventional methods (Table 1).[40] These Food and Drug Administration-cleared assays rapidly identify organisms growing in positive blood cultures, but do not eliminate the time required for growth from these cultures. Peptide nucleic acid fluorescent in situ hybridization molecular stains[41] is a well-validated method; the new QuickFISH system has reduced turnaround time to 20 min, enabling species identification results to be reported in the same time frame as Gram staining.[42] Polymerase chain reaction (PCR)-based methods, including GeneXpert (1 h), FilmArray (1 h), and Verigene (2.5 h), are somewhat slower than QuickFISH, but have little or no sample processing and include selected antibiotic resistance genes.[40] Rapid assays are gradually becoming less labor-intensive and have led to improved clinical outcomes, shorter hospital stays, and dramatically lower healthcare costs.[43,44]

Recent advances in molecular techniques enable amplification of microbial pathogens directly from whole-blood samples in under 12 h without relying on initial microbial growth in blood cultures (Table 1).[40] This provides the advantage of same-day identification and early targeted pathogen-specific antimicrobial therapy, especially in settings where there is pretreatment with antibiotics, low-density bacteremia, or where culture-negative sepsis is common. These molecular techniques predominantly rely on the amplification methods of PCR for the bacterial 16S or 23S ribosomal RNA (rRNA) genes and the 18S rRNA gene of fungi. Diagnostic accuracy of systems such as SeptiFast, SepsiTest, and, most recently, detection of PCR-amplified pathogen DNA from the blood that is hybridized to capture probe-decorated nanoparticles detectable by a small portable T2 magnetic resonance (MR) platform have been reported.[45–48] The Roche Light Cycler SeptiFast system requires 100 µL of blood and can detect 25 pathogens known to cause >90% of bloodstream infections, with a turnaround time of 6 h. A competing commercial assay, SepsiTest, is able to detect >300 pathogens; however, with a relatively slower turnaround time of 8–12 h.[46] The T2 MR is an automated nanoparticle-based PCR assay that can detect as few as 1 CFU/mL of *Candida* spp. in the blood in ~3 h.[46]

Some studies report a discordance between conventional culture and PCR methods during validation of molecular pathogen detection methods, which has led to continued uncertainty about the bacterial etiology of sepsis.[49,50] Furthermore, false-positive results were seen with high cycle thresholds, thus opening the possibility for nonspecific amplification and raising the questions about whether the bacteria present was the cause for the sepsis syndrome.[51] A systematic review concluded that molecular diagnostics had value as adjunctive tests with an overall sensitivity of 90% and specificity of 96%.[52] Molecular assays are not readily available, may be expensive, and have modest diagnostic accuracy. Hence, molecular assays are not ready to replace blood cultures as reference standards, but are useful as adjunctive tests in the diagnosis of neonatal sepsis.

Hematological indices

Leukocyte (<5000 or ≥20000/mm^3), absolute neutrophil (<1000 or ≥5000/mm^3) and immature/total neutrophil counts (>0.2), and peripheral blood smear (toxic granulation, vacuolization and

Table 1. Microbial identification from blood cultures and molecular nonculture techniques.

Technique	Target pathogen	Resistance typing	Turnaround time	Sensitivity	Specificity
Culture-based technique					
Blood culture	All culturable microbes	Yes	48–72 h	—	—
Automated identification	All culturable microbes	Yes	24–48 h	—	—
Nucleic acid-based identification					
PNA-FISH	Differentiates between *Staphylococcus aureus* and CoNS; *Enterococcus faecalis* and *Enterococcus* species; *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*; and *Candida* species	No	1.5–3 h[a]	96–100%	96–100%
*Quick*FISH	*S. aureus*, CoNS, *E. faecalis*, other Enterococci, *E. coli*, *K. pneumoniae*, *P. aeruginosa*	No	<30 min [a]	96–100%	96–100%
MALDI-TOF	GP and GN bacteria, yeast, fungi, filamentous fungi, mycobacteria	In development	10–30 min [a]	—	—
GeneXpert MRSA/SA	*S. aureus*	mecA for methicillin resistance	<1 h[a]	98.3–100% for MSSA and MRSA	98.6–99.4% for MSSA and MRSA
Verigene gram-positive	*Staphylococcus* spp., *S. aureus*, *S. epidermidis*, *S. lugdunensis*, *Streptococcus* spp., *S. pyogenes*, *S. agalactiae*, *S. anginosus* group, *S. pneumoniae*, *E. faecalis*, *E. faecium*, and *Listeria* spp.	*mecA* for methicillin resistance and *vanA/B* genes for vancomycin resistance	2.5 h[a]	92.6–100%	95.4–100%
Verigene gram-negative	9 bacterial targets including *E. coli*, *Shigela* spp., *K. pneumoniae*, *K. oxytoca*, *P. aeruginosa*, *Serratia marcescens*, *Acinetobacter* spp., *Proteus* spp., *Citrobacter* spp., *Enterobacter* spp.	KPC, NDM, CTX-M, VIM, IMP, OXA	2 h[a]	97.1%	99.5%
FilmArray	27 targets, including staphylococci, streptococci, *Enterococcus*, *Listeria*, *Acinetobacter*, *Neisseria meningitidis*, *P. aeruginosa* and members of the Enterobacteriaceae family, as well as *Candida* spp.	*mecA*, *vanA/B*, and *K. pneumoniae* carbapenemase (KPC) genes	1 h[a]	>90%	—
Culture-independent diagnostic tests					
SeptiFast	25 pathogens (10 GN, 9 GP, 6 fungi)	—	4–6 h	63–83%	83–95%
SepsiTest	>345 pathogens, 13 fungi	—	8–12 h	11–87%	83–96%
T2 MR *Candida*	*C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*	In development	3–6 h	90%	98%

CONS coagulase-negative staphylococci, *GP* gram positive, *GN* gram negative.
[a]Turnaround times are after culture turns positive.

Dohle bodies) are traditionally used to aid the diagnosis of neonatal sepsis (Table 2).[53]

White blood cell count (WBC). Leukocyte count starts between 6000 and 30,000/mm^3 in the first day of life and decreases to 5000–20,000 mm^3 later. Neutrophil count tends to be lower at lower gestational ages (GAs) and peaks 6–8 h after birth.[54] Clinical conditions such as maternal fever and hypertension, perinatal asphyxia, meconium aspiration syndrome, delivery route, intraventricular hemorrhage, hemolysis, pneumothorax, convulsion, and even crying affect the neutrophil count.[55] A literature review by Sharma et al. reported that leukopenia (WBC count <5000/mm^3) has a low sensitivity (29%), but high specificity (91%) for the diagnosis of neonatal sepsis.[56] Additional studies highlighted that leukopenia is more predictive of sepsis than leukocytosis (WBCs >20,000/mm^3) at >4 h.[57] Neutrophil/lymphocyte ratios (NLR) of 1.24:6.76 and platelet/lymphocyte ratios of 57.7:94.05 may be diagnostic of neonatal sepsis.[58,59]

Absolute neutrophil count (ANC). Neutrophil counts are commonly evaluated in neonates with presumed sepsis, but can be affected by maternal and infant risk factors.[54,55] Neutropenia (ANC < 1000/mm^3 at ≥4 h) is considered more specific for early-onset neonatal sepsis (EOS) as opposed to neutrophilia (ANC ≥ 10,000/mm^3).[51,56,60] Interpretation of ANC, however, must take into consideration the neonate's gestational and postnatal age as the lower limit of ANC decreases with lower GA. Furthermore, an analysis of 30,354 complete blood counts (CBCs) obtained in the first 72 h of life demonstrated that ANC peak later in early preterm neonates <28 weeks gestation as compared with neonates ≥28 weeks gestation (24 h of life vs 6–8 h, respectively).[54] Mean neutrophil volume >157 arbitrary units had sensitivity and specificity as 79% and 82% while sensitivity and specificity of CRP were 72% and 99%, respectively.[61] In 141 neonates with neonatal sepsis, cut-off level of delta neutrophil index was calculated as 4.6% with 85% sensitivity and 80% while CRP had 81% sensitivity and 82% specificity.[62]

Immature-to-total neutrophil (I:T) ratio. Compared to other hematological markers, I:T ratio may be the most sensitive indicator of neonatal sepsis,[60] but this parameter also varies with GA and postnatal age. In healthy newborns, the I:T ratio peaks at 0.16 during the first 24 h and gradually declines over days. Gandhi and Kondekar propose that I:T ratio >0.27 in term newborns and >0.22 in preterm neonates favor the diagnosis of neonatal sepsis.[60] Murphy and Weiner demonstrated that two normal I:T ratios correlated with a sterile blood culture had a maximum NPV of 100%.[63]

Red cell distribution width (RDW). RDW shows increased red blood cell production in inflammatory and infectious diseases. Elevated RDW has been shown to be associated with increased mortality from sepsis in both adults and neonates.[64,65] In neonates, RDW was significantly higher in sepsis and among non-survivors.[66] Cut-off levels as 16.3 and 19.5 had sensitivity (70–87%) and specificity (66.1–81%) in neonatal sepsis and gram-negative late-onset neonatal sepsis (LOS), respectively.[64,67]

Thrombocytopenia. Thrombocytopenia is associated with neonatal sepsis.[68] Platelet volume increases while being more active and associated with cytokines and inflammatory mediators. A meta-analysis that included 11 studies and 932 patients reported that MPV was higher in neonatal sepsis with a cut-off level between 8.6 and 11.4.[69–71]

Inflammatory biomarkers

Acute-phase reactants. APRs are produced by the liver in response to cytokines, which are induced by infection and tissue injury. TNF-α, CRP, PCT, fibronectin, haptoglobin, pro-adrenomedullin (pro-ADM), and SAA have been evaluated in neonatal sepsis.

C-reactive protein: CRP has been the most studied biomarker.[16] Serum CRP concentrations rise within 10 to 12 h in response to bacterial infections and peak after 36–48 h, with concentrations that correlate with illness severity.[72] Due to the delay in elevation, it is unreliable for the early diagnosis of neonatal sepsis (low sensitivity).[15] Furthermore, other noninfectious maternal and neonatal conditions may also result in elevated CRP levels, thus making it a nonspecific biomarker.[72,73] A systematic review of biomarkers for neonatal sepsis concluded that serial measurements of CRP at 24 to 48 h after onset of symptoms has been shown to increase its sensitivity and NPV and may be useful for monitoring response to treatment in infected neonates receiving antibiotics.[16] This suggests that CRP may be more useful for ruling out infection and discontinuing antibiotics when serial measurements are obtained.

Procalcitonin: PCT is synthesized in monocytes and hepatocytes as a prohormone of calcitonin in response to cytokine stimulation. After birth, it increases until postnatal day 2–4.[74] PCT is downregulated by IFN-γ, a commonly produced cytokine in viral infections.[72,75,76] Thus, PCT has emerged as a promising biomarker for the diagnosis of bacterial infections that may be useful in discriminating between bacterial and viral etiologies. After exposure to bacterial endotoxin, PCT levels rapidly rise within 2–4 h and peak within 6–8 h, thus making it a more sensitive marker than CRP for early diagnosis of neonatal sepsis.[77] This increase often correlates with the severity of the disease and mortality. However, in EOS, PCT measurements at birth may initially be normal; a serial PCT measurement at 24 h of age may be more helpful for early diagnosis.[78] Furthermore, serial PCT determinations allow shortening the duration of antibiotic therapy in term and near-term infants with suspected early-onset sepsis.[79] However, before this PCT-guided strategy can be recommended, its safety and reliability must be confirmed in a larger cohort of neonates.

In a meta-analysis with 1959 patients, sensitivity and specificity of PCT were reported to be 81% (95% confidence interval (CI): 74–87) and 79% (95% CI: 69–87), respectively.[80] Studies in the meta-analysis used different cut-off thresholds (0.8–2.4 μg/L). PLR and NLR were 7.7 and 0.11 for LOS, while 3.2 and 0.3 for EOS indicating that diagnostic accuracy is better in LOS.[81] Cord blood PCT >0.7 μg/L in the diagnosis of sepsis showed 69% sensitivity and 70% specificity and PCT has been used in combination with other biomarkers in EOS.[82] Canpolat et al. reported that PCT (>1.74 ng/mL) and CRP (>0.72 mg/dL) had 76% and 58% sensitivity and 58% and 85% specificity, respectively, on the third day of life in neonates with preterm premature rupture of membranes.[83] Eschborn and Weitkamp evaluated 29 studies comparing PCT with CRP and found that mean sensitivity for EOS, LOS, and EOS + LOS was 73.6%, 88.9%, and 76.5% for PCT; 65.6%, 77.4%, and 66.4% for CRP, while mean specificity for EOS, LOS, and EOS + LOS was 82.8%, 75.6%, and 80.4% for PCT; 82.7%, 81.7%, and 91.3% for CRP, respectively.[72] The authors concluded that the performance of both biomarkers will be better with serial measurements, and correlation with clinical findings is needed for decision-making.

Serum amyloid A: SAA is another APR synthesized by hepatocytes, monocytes, endothelial, and smooth muscle cells in 8–24 h after bacterial exposure and is regulated by pro-inflammatory cytokines. SAA levels increase with age, with the lowest levels seen in umbilical cord blood, and the highest levels seen in old age.[84] In response to infection or injury, SAA levels rapidly increase up to 1000 times higher than baseline, but can be significantly influenced by the patient's hepatic function and nutritional

Table 2. Most studied and promising biomarkers in the diagnosis of neonatal sepsis.

Biomarker	Patient characteristics	Performance							Comments
		Cut-off	Sensitivity	Specificity	PPV	NPV	PLR	NLR	
Complete blood cell count[51]									
WBC	EOS LOS	—	0.3–18 0.1–23	79–99 80–99	36 13–100	94–>99.8 74–96	—	—	Leukocyte (<5000, ≥20,000) and absolute neutrophil count (<1000, ≥5000) were traditionally used parameters, but these parameters are affected by gestational age, postnatal hours and days, and clinical conditions such as maternal hypertension, perinatal asphyxia, intraventricular hemorrhage, etc.
ANC	EOS LOS	—	8–68	95–99	14–21	74–96	—	—	
I/T ratio	EOS LOS	—	22–62 33–54	74–96 62–100	2.5 12–100	99 66–96	—	—	The I/T ratio has better sensitivity than WBC and ANC Disadvantage of this ratio is the interreader difference. The ratio >0.2 has been traditionally used
Platelet count	EOS LOS	—	0.8–4 8–48	97–99 89–98	13–14 99	— 94	—	—	Low platelet count can be found in neonatal sepsis, especially gram-negative and fungal sepsis, but often remain decreased during the sepsis process
MNV, MNC, MNS	76 proven, 126 clinical sepsis, 98 control All gestations (mean 30 ± 5 w), early- and late-onset sepsis[61]	>157 a.u. <159 a.u. <127.5 a.u.	79 66 60	82 64 65	90 80 21	65 47 55	—	—	No statistical difference between early- and late-onset sepsis; proven and clinical sepsis. Combination with IL-6 and CRP gave a better diagnostic performance. Levels normalized with treatment
DNI	110 proven, 31 clinical sepsis, 87 control All gestations (median 30 (23–41) w), early- and late-onset sepsis[62]	4.6	85	80	87	77	—	—	DNI was insignificantly higher in late-onset sepsis. Proven sepsis had significantly higher DNI levels. Levels normalized with treatment. Mortality was predicted with DNI
CD64	Meta-analysis of 17 studies including 3478 neonates, all gestations, EOS and/or LOS, proven and/or clinical sepsis[118]	1.8–4.3 CD64 index; 1010–6010	21–100	59–100	9–96.2	73–100	1.84–47.1	0.06–0.48	Pooled sensitivity, specificity, PLR and NLR were 77%, 74%, 3.58, and 0.29, respectively. The pooled DOR was 15.18 (95% CI: 9.75–23.62). The proven sepsis group had better diagnostic performance than the clinical sepsis group. Term infants had higher sensitivity, specificity, PLR, and DOR

Table 2 continued

Biomarker	Patient characteristics	Performance							Comments
		Cut-off	Sensitivity	Specificity	PPV	NPV	PLR	NLR	
CD11b	Meta-analysis of 9 studies including 843 neonates, all gestations, EOS and/or LOS, proven, and/or clinical sepsis[121]	12.6–600 MFI	65–100	56–100	50–100	61–100	2.1–156	0.01–0.49	Pooled sensitivity, specificity, PLR and NLR were 82%, 93%, 11.51 and 0.19, respectively. The pooled DOR was 59.50 (95% CI: 4.65–761.58). The diagnostic accuracy was higher in early-onset sepsis
Presepsin	Meta-analysis of 11 studies including 793 neonates, all gestations, EOS and/or LOS, proven and/or clinical sepsis[122]	≤650 650–850 ≥850	91 91 90	85 97 86	—	—	—	—	The pooled DOR: 71.78 (7.46–690.56) 542.72 (156.62–1880.60) 75.60 (8.32–686.53)
	Meta-analysis of 28 studies including 2661 neonates, all gestations, EOS and/or LOS, proven and/or clinical sepsis[77]		67–98	75–100	—	—	—	—	Pooled sensitivity, specificity, PLR and NLR were 85%, 98%, 50.8 and 0.06, respectively. The pooled DOR was 864
sTREM-1	Meta-analysis of 8 studies including 667 neonates, all gestations but mostly term infants, EOS and/or LOS, proven and/or clinical sepsis[128]	77.5–1707.35 pg/mL	70–100	48–100	34–93.3	62–90	1.6–9.33	0.07–0.48	Pooled sensitivity, specificity, PLR and NLR were 95%, 87%, 7 and 0.05, respectively. The pooled DOR was 132.49 (95% CI: 6.85–2560.70)
IL-6	Meta-analysis of 31 studies including 1448 septic neonates[102]	3.6–300 pg/mL	54–100	45–100	—	—	1.63–88.79	0.03–0.50	Pooled sensitivity, specificity, PLR and NLR were 88%, 82%, 7.03 and 0.2, respectively. The pooled DOR was 29.54 (95% CI 18.56-47.04)
IL-8	Meta-analysis of 8 studies including 548 neonates, all gestations, EOS and/or LOS, proven and/or clinical sepsis[109]	0.65–100 pg/mL	34–94	66–100	64–100	59–95	2.22–80.49	006–0.76	Pooled sensitivity, specificity, PLR and NLR were 78%, 84%, 4.58 and 0.25, respectively. The pooled DOR was 21.64 (95% CI: 7.37–63.54)
TNF-α	Meta-analysis of 15 studies including 1201 neonates[111]	0.18–180 (20,000 in 2 studies)	21–100	43–100	—	—	—	—	Pooled sensitivity and specificity were 66%, 76%, respectively. The pooled DOR was 7.43 (95% CI 3.47–15.90). Diagnostic accuracy was found slightly better in LOS than EOS
CRP	Review of 27 studies including 4996 neonates[112]	2.5–100 mg/L	22–100	59–100	31–100	38–96	—	—	
PCT	Meta-analysis of 16 studies including 1959 neonates, all gestations, EOS and/or LOS, proven and/or clinical sepsis[80]	0.5–5.75 µg/L	57–100	50–100	19–100	56–100	—	—	Pooled sensitivity, specificity, PLR and NLR were 81%, 79%, 3.9 and 0.24, respectively. The pooled DOR was 16 (95% CI: 8–32). Diagnostic accuracy was better in LOS than EOS
SAA	Meta-analysis of 9 studies including 823 neonates, all	1–68 mg/L	23–100	33–100	57–100	57–100	—	—	Pooled sensitivity and specificity were 84%, 89%, respectively. The pooled DOR was 91.84 (95%

Table 2 continued

Biomarker	Patient characteristics	Performance							Comments
		Cut-off	Sensitivity	Specificity	PPV	NPV	PLR	NLR	
	gestations, EOS and/or LOS, proven and/or clinical sepsis[87]								CI: 16.78–502.80). CRP has higher pooled sensitivity and DOR than SAA
Pro-ADM	31 proven, 41 clinical sepsis and 52 control, preterm and term infants, EOS and/or LOS[89]	3.9 nmol/L	86	100	100	83	—	—	Diagnostic accuracy of pro-ADM was similar to IL-6 and CRP. Higher pro-ADM levels were found in gram-negative sepsis
Hepcidin	27 neonates with LOS and 17 control, VLBW infants[94]	92.2 mg/dL	76	100	100	87	—	—	Diagnostic performance was better than CRP and combination with CRP did not give better performance than hepcidin alone
Progranulin	2 studies: neonates >34 w at risk of EOS, proven and clinical sepsis (*n*: 152), (*n*: 121)[95]	1.39–37.86 ng/ml	67–94	80–51	76–61	67–91	3.4–1.95	0.16–0.11	Progranulin was found efficient to predict EOS. Combination with CRP, PCT, and IL-6 gave a better diagnostic performance
Vascular endothelium	74 infected, 118 non-infected samples of 149 neonates, preterm, and term infants with EOS or LOS[99] sICAM-1 sE-Selectin SAA	228 ng/mL 132 mg/L 1 mg/L	76 54 23	75 82 92	66 66 66	83 73 66	—	—	LOS group had higher sICAM-1 and SAA while lower sE-Selectin levels. The combination of these biomarkers with hsCRP altogether gave sensitivity, specificity, PPV and NPV as 90%, 67%, 64 and 91%, respectively.
Molecular assays (Meta-analysis[52])	All molecular tests (35 studies) Broad range PCR (9 studies) Real-time PCR (9 studies) Post-PCR processing (5 studies) Multiplex PCR (6 studies) LOS (10 studies) EOS and LOS (23 studies) Preterm (5 studies) Preterm and term (30 studies)	—	90 (38–100) 97 86 97 76 79 94 89 90	93 (32–100) 93 94 96 81 84 92 87 94	—	—	—	—	Molecular tests have the advantage of rapid results and can be used as add-on tests. Molecular assays, including PCR and hybridization methods, and have rapid detection times compared to blood cultures (6-8 h vs 20-36 h). Costs, availability of equipment and need for technical skills are disadvantages.
Future									
Omics approach	Metabolomics: sugars, lipids, small peptides, vitamins including glucose, maltase, lactate, acetate, ketone bodies, D-serine, acylcarnitines, acetoacetate, creatine[146] Proteomics: neutrophil defensin 1–2, cathelicidin, S100A12, S100A8, proapolipoprotein C2, apolipoprotein A–E–H, β-2 microglobulin, haptoglobin, desarginin[141,142]								
Nanotechnology	Magnetic, gold, fluorescent, and lipid-based nanoparticles for contrast agents and biosensors[45,46]								
Machine learning									
Heart rate variability	Reduced heart rate variability and transient decelerations were associated with early diagnosis of sepsis in 633 neonates,[152] while reduced mortality due to different clinical problems including sepsis was reported in 2989 VLBW infants[151]								
Vital signs	Heart rate, respiratory rate, temperature, desaturations, bradycardias of 155 neonates between 23 and 32 w with LOS[153]	Triggering score ≥5	81	80	57	93	—	—	LOS was diagnosed 43.1 ± 79 h before culture positivity

Table 2 continued

Biomarker	Patient characteristics	Performance							Comments
		Cut-off	Sensitivity	Specificity	PPV	NPV	PLR	NLR	
Clinical findings	Fever, apneas, platelet counts, gender, bradypnea, band cells, catheter use, birth weight and maternal age, cervicovaginitis in 238 neonates[154]	—	93	80	82	92	—	—	25 potential maternal and neonatal features were studied. The predictive model was created with a combination of clinical, laboratory, and demographic features
	Blood pressure, temperature, and saturation were found as vital candidate markers out of heart rate, respiratory rate, systolic blood pressure, and diastolic blood pressure in 7870 neonates[155]								
New genetic techniques	Noncoding RNAs: miRNA, circRNA,	miRNA-181a[157] miRNA-16a[158] miRNA-451[158]	0.625 3.1 1.2	83 88 64	84 98 60	— 95 61	— 88 62	—	—	Study groups included term infants, both EOS and LOS, proven and clinical sepsis. miRNA levels were correlated with WBC, CRP, and respiratory discomfort

WBC white blood cell count, *EOS* early-onset neonatal sepsis, *LOS* late-onset neonatal sepsis, *ANC* absolute neutrophil count, *I/T ratio* immature/total neutrophil count ratio, *MNV* mean neutrophil volume, *MNC* mean neutrophil conductivity, *MNS* mean neutrophil scatter, *DNI* delta neutrophil index, *CD* cluster of differentiation, *sTREM-1* soluble triggering receptor expressed on myeloid cells-1, *IL* interleukin, *TNF-α* tumor necrosis factor-α, *CRP* C-reactive protein, *PCT* procalcitonin, *SAA* serum amyloid A, *pro-ADM* proadrenomedullin, *sICAM-1* soluble intercellular adhesion molecule-1, *sE-Selectin* soluble endothelial leukocyte adhesion molecule-1, *PCR* polymerase chain reaction, *VLBW* very low birth weight infants, *RNA* ribonucleic acid, *miRNA* microRNA, *circRNA* circular RNA, *NET* neutrophil extracellular traps, *PPV* positive predictive value, *NPV* negative predictive value, *PLR* positive likelihood ratio, *NLR* negative likelihood ratio, *DOR* diagnostic odds ratio, *MFI* mean fluorescence intensity. Antibody–phycoerythrin molecules bound per cell.

status.[85] In a study by Arnon and Litmanovitz, when compared with healthy infants at 0, 8, and 24 h, SAA levels in septic infants were significantly higher ($p < 0.01$) at all time points.[53] When compared with CRP, SAA had an overall better diagnostic accuracy for predicting EOS. Cetinkaya et al. also determined that SAA concentrations had better sensitivity and area under the curve when compared with CRP and PCT, although the difference was not statistically significant.[86] Different cut-off points between 1 and 68 mg/L were reported with a pooled 78% sensitivity and 92% specificity.[87]

Proadrenomedullin is a stable precursor of ADM, which modulates circulation, has antimicrobial properties, and protects against organ damage.[88] High sensitivity (86.8%), specificity (100%), PPV (100%), and NPV (83.9%) with a cut-off value of 3.9 nmol/L of pro-ADM were observed in 76 neonates with neonatal sepsis.[89] Higher pro-ADM levels were associated with increased sepsis severity and mortality.[90]

Adipokines are released from adipose tissue and may initiate the secretion of inflammatory and anti-inflammatory cytokines. Visfatin (>10 ng/mL) and resistin (>8 ng/mL) had sensitivity and specificity over 90% in 62 septic neonates.[91] Subsequent studies reported lower sensitivity and specificity for resistin, but levels were positively correlated with IL-6 and CRP.[92,93] Hepcidin, progranulin, stromal cell-derived factor 1, endocan, and pentraxin-3 are less studied APRs, which have a role in inflammation, chemoattraction, complement activation, angiogenesis, and future studies are needed to evaluate the diagnostic accuracy of these markers.[94–98]

Vascular endothelium: Vascular endothelium interacts with leukocytes, soluble mediators, PAMPs, and DAMPs, which have a role in sepsis pathogenesis. E-selectin, L-selectin, soluble intracellular adhesion molecule-1, soluble vascular cell adhesion molecule-1, and angiopoietin 1–2 were studied in the diagnosis of neonatal sepsis.[99] However, the limitation of these markers includes no normative data in neonates, physiological increase in the first month of life, and lack of large studies.

Interleukins: IL-6 increases immediately after exposure to pathogens and normalizes in 24 h.[100] IL-6 has a proinflammatory effect inducing CRP, fibronectin, and SAA release from the liver, T cell differentiation, and B cell maturation.[101] IL-6 has been studied more than other cytokines and found to be increased in neonates with EOS and LOS, and various cut-off levels between 18 and 300 pg/mL were reported in 31 studies with 1448 septic neonates.[102] The pooled sensitivity and specificity of IL-6 were 88% and 82%, while PLR and NLR were 7.03 and 0.2, respectively. The combination of IL-6 with other markers such as CRP, pro-ADM, and PCT showed better diagnostic accuracy.[19,89,103]

Cortes et al. evaluated the diagnostic accuracy of IL-6 and CRP in EOS and LOS.[104] The authors concluded that IL-6 (>17.75 pg/mL) showed greater accuracy in EOS, while CRP (>0.53 mg/dL) was more accurate in LOS. Kurul et al. showed that IL-6 (>580 pg/mL) and PCT (>0.94 ng/mL) were associated with 7-day mortality, while CRP was not.[105]

Ye et al. evaluated the utility of cytokines in 420 neonates with neonatal sepsis.[106] Interleukin-2, IL-4, IL-6, IL-10, TNF-α, and INF-γ were measured and compared with CRP. Interleukin-6 (>12.5 pg/mL) and IL-6/IL-10 ratio (>3.5) were found as valuable as CRP, while most sensitive and specific ILs were IL-6 (94.1%) and IL-6/IL-10 ratio (100%), respectively. Celik et al. observed that a cut-off level of 202 pg/mL for IL-6 differentiated gram-negative ($n = 73$) from gram-positive ($n = 82$) sepsis with 68% sensitivity and 58% specificity.[107] In a later study, IL-6 (>400 pg/mL) alone or in combination with TNF-α (>32 pg/mL), IL-8 (>200 pg/mL), and granulocyte-colony stimulating factor (>1000 pg/mL) had 100% sensitivity, specificity, NPV and 38–69% PPV to differentiate gram-negative neonatal sepsis.[108]

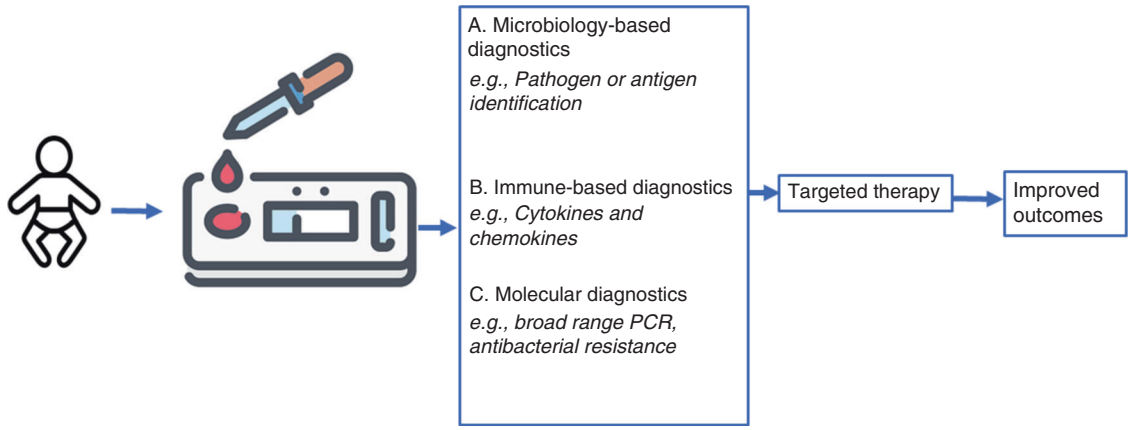

Fig. 3 Point of care testing for the diagnosis of neonatal sepsis. Blood samples are drawn on suspicion of infection on laboratory chips that are microbiology, immune, or molecular-based diagnostics. The results enable us to initiate targeted therapy. The rapid results and targeted therapy will improve clinical outcomes.

IL-8 is another proinflammatory cytokine promoting chemotaxis and activation of granulocytes and increases within 1–3 h with a half-life <4 h. Diagnostic accuracy was evaluated in a meta-analysis with eight studies with 548 neonates (cut-off levels between 0.65 and 300 pg/mL), which reported a pooled sensitivity and specificity of 78 and 84% similar to CRP.[109]

TNF-α is secreted from natural killer cells by IL-2 to induce T cell proliferation, vasodilatation, and neutrophil adhesion.[110] In a systematic review (where TNF-α cut-off values ranged from 1.7 to 70 pg/mL) at a mean cut-off value of 18.94 pg/m/L, the sensitivity was 79% and specificity was 81% and better accuracy in LOS than EOS.[111] Meta-analyses of data from neonates show variable sensitivity and specificity for IL-6, IL-8, and TNF-α with only moderate accuracy in diagnosing neonatal sepsis.[16,109,111] However, when combined with other cytokines or late pro-inflammatory markers, such as CRP, sensitivity, and specificity increase.[106,112,113] Currently, measuring cytokines for the diagnosis of neonatal sepsis may not be practical or cost-effective because enzyme immunoassays are expensive and time-consuming.

Cell adhesion molecules: Leukocyte antigens are upregulated after bacterial exposure and can be quantified by flow cytometry.[114,115] These markers increase in minutes after infection and levels were not affected by GA, the timing of sepsis onset, type of microorganism, or non-infectious diseases.[116,117] Limitation of these markers is in need of high technology and non-standardized normal ranges.

Cluster differentiation molecule-64 (CD64) expressed from neutrophils and monocytes facilities phagocytosis and intracellular killing of opsonized microorganisms. Increased levels can be detected in 1 h and stable for 24 h. Shi et al. performed a meta-analysis of CD64 levels from 17 studies, including 3478 neonates, and found that pooled sensitivity, specificity, PLR, and NLR were 77%, 74%, 3.58, and 0.29, respectively.[118] Serial measurements and combination with other markers have been reported with varying diagnostic accuracy.[119,120] Increased CD11b expression was found both in EOS and LOS with high sensitivity and specificity up to 100%.[112] In a recent meta-analysis including nine studies with 843 neonates showed that CD11b is a promising biomarker with sensitivity, specificity, PLR, and NLR as 82%, 93%, 11.51, and 0.19, respectively.[121]

Soluble CD14 fragment (presepsin) is a specific and high-affinity receptor complex of LPSs and activates TLR to proinflammatory cytokine secretion. Both meta-analyses revealed that presepsin was as accurate as PCT and CRP in the diagnosis of neonatal sepsis.[77,122] Gram-negative infections lead to higher sCD14 levels.[123] Cord blood presepsin levels were evaluated in 288 preterm infants with premature rupture of membranes for EOS and a cut-off level ≥1370 pg/mL yielded an odds ratio of 12.6 (95% confidence level: 2.5–28.1).[124] Presepsin, PCT, IL-6, and IL-8 were compared in the diagnosis of EOS and presepsin was found as the most accurate biomarker with 88.9% sensitivity and 85.7% specificity.[125]

Soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) regulates the innate immune system and inflammation by promoting the release of proinflammatory cytokines. Increased levels were found in neonatal sepsis with a cut-off value of 310 pg/mL, although higher levels were reported in culture-proven sepsis.[126] Urine sTREM-1 >78.5 pg/mL had 90% sensitivity, 78% specificity, 68% PPV, and 94% NPV in 62 neonates with sepsis, respectively.[127] A meta-analysis including eight studies with 667 neonates reported that sensitivity and specificity of sTREM-1 were 95% and 87%, respectively.[128] Limitations include a small number of studies and different cut-off levels between 77.5 and 1707 pg/mL.[128]

The challenge of biomarker identification is reflected by the fact that over 3000 sepsis biomarker studies have been published with almost 200 candidate biomarkers evaluated.[129] However, there is not a single biomarker that has sufficient diagnostic accuracy for the diagnosis of neonatal sepsis. The combination of biomarkers or their serial measurements may be strategies to enhance diagnostic accuracy. A combination of IL-6, sTREM-1, and PCT has been suggested, as each biomarker represents a different component in the pathophysiology of sepsis.[130] Others propose that early- and mid-phase markers such as neutrophil CD64 and PCT should be combined with the late-phase biomarker CRP for maximal diagnostic benefit.[40] A recent literature review summarizes the utility of combining both early and late biomarkers for neonatal sepsis.[130]

Strategies for the future

Mass spectrometry for identification of pathogens from blood culture specimens. Matrix-assisted laser desorption-ionization/time-of-flight (MALDI-TOF) mass spectroscopy is a relatively new approach that can identify microorganisms within 30 min after blood culture positivity.[131]Meta-analyses have found that the use of MALDI-TOF for diagnosis of infection from culture bottles has acceptable sensitivity and specificity[132] and with higher sensitivity in gram-negative infections compared to gram-positive infections.[133]

Point-of-care (POC) devices for diagnosis of neonatal sepsis. Rapid tests done at the bedside that could confirm the diagnosis or provide prognostic information have the potential to improve patient outcomes and decrease healthcare costs (Fig. 3). Novel techniques such as analysis of volatile organic compounds in the

breath have been demonstrated to be reasonably sensitive and specific[134] and capable of distinguishing sepsis from inflammation in rat models,[135] yet to be validated in human studies. POC devices using a variety of biomarkers including blood plasma protein quantification and leukocyte monitoring are being evaluated for the diagnosis of sepsis.[136]

Omics technologies and personalized medicine. Omics technologies provide data on genome-wide gene expression, protein translation, and metabolite production that are differentially regulated in neonatal sepsis.[137,138] Proteomics measures protein components released after infection or inflammation. Cord blood and amniotic fluid proteomics have provided information regarding the fetal response to intra-amniotic inflammation and have successfully predicted EOS with >92% accuracy.[139,140] Proteomics including neutrophil defensin 1–2, cathelicidin, S100A12, S100A8, pro-apolipoprotein C2, apolipoprotein A-E-H, β-2 microglobulin, haptoglobin, desarginin from amniotic fluid, cord blood, and plasma were found to be valuable in the diagnosis of EOS and LOS.[141–143]

Metabolomics by nuclear MR imaging (NMR) and gas chromatography-mass spectrometry (GC-MS) has also been investigated in adult sepsis with favorable results.[144] Urinary metabolomics profile of adult pneumococcal pneumonia, for example, has been found to be distinctly different from viral and other bacterial causes of pneumonia.[145] This indicates that evaluation of urinary metabolite profiles may be useful for effective diagnosis and lead to faster targeted antibiotic treatment. Urine samples of neonates with sepsis were evaluated with H-NMR and GC-MS showed an increase in glucose, maltose, lactate, acetate, ketone bodies, D-serine, and also normalization of variations with treatment.[146]

A prospective observational study comparing genome-wide expression profiles of 17 VLBW infants with bacterial sepsis identified distinct clusters of gene expression patterns in gram-positive and gram-negative sepsis when compared with controls.[147] Genomic analysis may determine sepsis risk, treatment response, and prognosis while evaluating gene variants responsible for PRPs, signaling molecules and cytokines.[143,148]

Machine learning. Machine learning and artificial intelligence are increasingly used to sort transcriptomic, proteomic, and metabolomic data for biomarker screening, developing prognostication models, and for identifying the right patients for specific therapies (personalized medicine). One example is the Pediatric Sepsis Biomarker Risk Model (PERSEVERE), which was developed and validated as a prognostic enrichment tool for pediatric septic shock and in predicting mortality.[138,149] Ongoing research is investigating the application of the PERSEVERE model in neonatal sepsis prognostication.[150]

Reduced heart rate variability and transient decelerations were detected in hours to days before diagnosis of sepsis.[151,152] In these studies, early diagnosis of sepsis and reduced mortality has been reported. Recently predictive models using machine learning were developed. These models use the vital signs, clinical, and laboratory features of patients. Mithal et al. calculated a triggering score of ≥5 by using heart rate, respiratory rate, temperature, desaturation, and bradycardia events. The authors found that LOS was diagnosed 43.1 ± 79 h before culture positivity with 81% sensitivity, 80% specificity, 57% PPV, and 93% NPV in 72 patients.[153] Clinical findings such as birth weight, gender, catheter use, and laboratory findings such as blood gas parameters, CBC were also integrated into prediction models and found valuable in the diagnosis of sepsis.[154,155]

New genetic techniques. Non-coding RNAs (transcriptomics) including microRNAs (miRNA) and circular RNAs regulate many cell signaling pathways including cell proliferation, differentiation, development, metabolism, apoptosis, and proinflammatory cytokine production.[156] Both increased (miRNA 15-16a-23b-451) and decreased (miRNA 25-129-132-181a-223) expression were reported, while 80–89% sensitivity and 79–98% specificity were found in the diagnosis of neonatal sepsis.[157,158] Exosomes and neutrophil extracellular traps released during inflammation may be therapeutic targets in the future.

Conclusions

Identification of an ideal biomarker to diagnose neonatal sepsis is still the holy grail, but advances in technology have given us a glimpse of the promising tests for the future. Inflammatory markers such as CRP and PCT as well as other hematological indices used currently have limited value in neonates. Serial measurements of an ideal combination of biomarkers have shown to increase diagnostic accuracy but remain expensive and cumbersome for clinical practice. Molecular diagnostic tools such as PCR and sequencing, as well as MS, offer promise for more rapid and sensitive detection of disease. Omics technology and machine learning may provide us with diagnostic and prognostic models that could be personalized for the future.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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