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Biomarkers for Late-Onset Neonatal Sepsis: Cytokines and Beyond

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KEYWORDS

• Biomarkers • Infection • Infants • Late-onset

Early and accurate diagnosis of late-onset neonatal sepsis (LONS) is a major diagnostic challenge in neonatology.[1,2] LONS occurs most frequently in preterm and very low-birth-weight (VLBW) infants and in newborns with surgical conditions that require prolonged parenteral nutrition and hospitalization in the neonatal intensive care unit (NICU). A recent multicenter survey suggests that more than one-fifth (21%) of VLBW infants have at least 1 episode of late-onset culture-proven sepsis.[2] To date, clinical differentiation between LONS, including septicemia, meningitis, and systemic infection/inflammation (eg, necrotizing enterocolitis [NEC]), and noninfectious conditions (eg, acute exacerbation of bronchopulmonary dysplasia, apnea of prematurity, and gastrointestinal dysmotility) remains difficult, if not impossible, at an early stage of the illness.[1,3] A test or biomarker, which can accurately identify active infection/inflammation including septicemia and NEC in these vulnerable patients, would provide invaluable information for diagnosis and management. This review focuses on (1) the properties of an ''ideal'' diagnostic marker (or panel of biomarkers) of infection, (2) different categories of inflammatory mediators, such as acute phase proteins, chemokines, cytokines, and cell-surface antigens, that could potentially be used as clinical biomarkers, and (3) the use of molecular and biogenetic techniques for identification of pathogens in sterile body fluids. The authors also discuss recent scientific advances to search for novel biomarkers of infection in newborns.

THE IDEAL BIOMARKER OR TEST FOR LONS

The authors have previously proposed a set of clinical and laboratory criteria to assist neonatologists in identifying the ideal diagnostic marker of infection.[1] Although the

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fundamental principles remain unchanged, with continuing advances in technology, neonatologists now expect more clinical information to be provided by biomarkers. **Box 1** summarizes current views on the characteristics of the ideal biomarker. The biomarker should not only serve as a guide on when to stop antimicrobial treatment in noninfected infants but also aid in the decision of whether to start antibiotic treatment at the onset of nonspecific clinical signs. With advances in molecular biogenetic techniques, the ideal biomarker or test is also expected to pinpoint precisely the identity or category of microorganism causing sepsis.[4] Information on the severity of infection and likelihood of progression to disseminated intravascular coagulation (DIC) would also provide invaluable insights to clinicians for targeting infants with sepsis who are most in need of urgent treatment and intensive care support.[5] Identification of the pathogen and antibiotic susceptibility profile at disease onset would also contribute enormously to acute management.

Box 1
The ideal biomarker or test for LONS

Clinical properties

1. Provide an algorithm for starting and/or stopping antimicrobial treatment. Such biomarkers should have
   - A well-defined cutoff value
   - A sensitivity and negative predictive value approaching 100% for "ruling out" LONS (but simultaneously having high specificity and positive predictive value >85%)

Note: A biomarker or test with very high specificity and positive predictive value can be used for ruling in sepsis

2. Detect infection early (ie, at clinical presentation)
3. Identify a specific pathogen or a category of pathogens (eg, viral, bacterial, and fungal organisms; gram-positive organisms vs gram-negative organisms; a specific species of pathogen)
4. Monitor disease progress and guide antimicrobial treatment (eg, bacterial antibiotic resistance gene detection)
5. Predict the disease severity at the onset of infection (eg, identify the type of virulent pathogen, predict DIC at the onset of disease presentation)
6. Predict prognosis (ie, mortality)

Laboratory properties

1. Stable compound that may allow an adequate time window for specimen collection within normal working hours (ie, sustained increase or decrease in biomarker level for at least 24 hours) or easy storage of the specimen without significant decomposition of the active compound until laboratory processing
2. Quantitative determination of biomarker concentration
3. Automatic and easy method of measurement
4. Quick turnaround time (ie, specimen collection, transport, laboratory processing time, and reporting of results to clinicians within 6 hours)
5. Small volume of specimen (ie, <0.5 mL blood)
6. Daily or on-demand availability of testing in clinical laboratories
7. Low-cost test that can be used as a routine measurement

Biochemically, a biomarker should be stable and remain significantly upregulated or downregulated in the body fluid compartment for at least 12 to 24 hours even after commencement of appropriate antimicrobial treatment. This would increase the chance of a true positive test result, given the variability of timing of testing relative to onset of disease and treatment. The ideal biomarker would be resistant to decomposition during transport and storage so that the result would accurately reflect the infant's clinical condition at the time of specimen sampling. Other properties listed in **Box 1** are also crucial for maximizing the utility of a biomarker as a routine diagnostic test in the NICU setting.

NONSPECIFIC BIOMARKERS OF LONS

To date, most of the biomarkers investigated are key proinflammatory or antiinflammatory mediators of the infection/inflammatory cascade. A major disadvantage of nonspecific biomarkers is their tendency to be influenced by inflammatory conditions that are not induced by sepsis, such as tissue injury and surgery. Furthermore, localized infections frequently escape detection. The discovery of new biomarkers is key because blood culture, the current gold standard for diagnosing septicemia, is suboptimal in newborn infants. False-negative results are common because of the small volume of blood sample and the intermittent presence and low density of circulating pathogens during the early stages of infection. Pretreatment with antibiotics further exacerbates this problem. Hematologic tests, such as the total white cell count, differential white cell count, immature to total neutrophil (I/T) ratio, white cell morphology, platelet count, and various hematologic scores, are in general not considered to be particularly useful for differentiating between sepsis/NEC and noninfectious conditions.[6–8] These hematologic tests have insufficient sensitivity and specificity to guide clinical management, in particular, antibiotic treatment.[1] Hematologic tests such as the I/T ratio are also suboptimal because of their complex methodology and requirement of skillful technicians to identify immature neutrophil forms on a peripheral blood smear.[1,6] Although the presence of neutropenia and thrombocytopenia are suggestive of severe systemic infection, other noninfectious conditions such as severe lung disease can cause thrombocytopenia because of platelet sequestration.[9] Because hematologic parameters have limited diagnostic utility, recent studies of biomarkers have concentrated on 3 major categories of mediators: (1) acute phase proteins, (2) chemokines and pro- and antiinflammatory cytokines, and (3) cell-surface antigens.

Acute Phase Proteins

Of the acute phase proteins, C-reactive protein (CRP), serum amyloid A (SAA), and procalcitonin (PCT) are the most extensively studied. CRP is widely used in many NICUs for diagnosis and monitoring of treatment in LONS and NEC.[10–12] It has been previously reported that CRP is a late biomarker with high specificity for neonatal sepsis.[10] The concentration of serum CRP is usually not elevated at the time of clinical presentation but is delayed by 6 to 8 hours after onset of symptoms.[10] Although CRP is generally considered a nonspecific biomarker, research has shown that it has high specificity for neonatal systemic infection because preterm infants have a narrow spectrum of disease compared with older patients. Noninfectious inflammatory conditions that can confound the diagnosis of sepsis in adult patients, such as rheumatoid arthritis, other connective tissue diseases, and inflammatory bowel disease, occur rarely in neonates. Therefore, significant increases in serum CRP concentrations are more likely to be associated with systemic sepsis or bowel inflammation/necrosis secondary to NEC. Serial measurements of CRP concentrations are particularly useful

in ruling out sepsis, and persistently normal levels for 48 hours can assist in decision making for discontinuation of antibiotic treatment in infants with an equivocal presentation.[13] There are limitations to the application of measuring CRP levels because both false-positive and false-negative results have been reported. Importantly, the test is not sensitive in diagnosing localized infections such as pneumonia, urinary tract infection, and isolated low-grade fungal central nervous system infection.[14] It is also not a useful indicator after surgery or recent immunization because the circulating levels tend to be significantly elevated after these events.[1]

A study by Arnon and colleagues[15] comparing SAA with CRP and interleukin (IL)-6 suggested that SAA had higher sensitivity within the first 24 hours than the other 2 biomarkers for identification of LONS. In particular, the sensitivity of CRP was much worse during the early phase of infection, whereas IL-6 was suboptimal 24 hours after the onset.[10] The specificity of SAA was comparable to CRP throughout the clinical course. In addition, another study performed by the same investigators indicated that the mortality in infected preterm infants was inversely correlated with circulating SAA at 8 hours and at 24 hours.[16] These studies suggest that SAA may be a better biomarker than CRP and provide vital information on prognosis early in the course of infection.[16]

PCT has also been extensively studied in newborns and adults. The kinetics of PCT suggest that its serum concentration begins to increase 2 to 4 hours after exposure to bacterial products, peaks at 6 to 8 hours, and remains elevated for at least 24 hours. There is a physiologic increase in PCT levels during the first 48 hours of life, thought to be secondary to gastrointestinal bacterial colonization and subsequent translocation of endotoxin through the bowel wall.[17,18] However, the substantial increase in serum PCT concentration during bacterial infection can be easily differentiated from this minor physiologic increase during the immediate postnatal period.[18] Overall, the diagnostic utilities are similar to other acute phase reactants, although some studies have suggested that PCT may be superior with better sensitivity and specificity for identifying LONS.[18,19]

Other acute phase reactants and proteins, such as haptoglobin, lactoferrin, neopterin, inter-α-inhibitor proteins (IαIps),[20,21] lipopolysaccharide-binding protein (LBP),[22,23] and components of the complement pathways (eg, C5a, C5L2),[24,25] have been reported to be potentially useful diagnostic biomarkers. Particular interest has been focused on LBP and IαIps. LBP can theoretically fill in the diagnostic gap between the early (eg, IL-6) and the late biomarkers (eg, CRP) because of its chemical kinetics. A recent study has demonstrated that LBP was superior in sensitivity and negative predictive value compared with PCT, IL-6, and CRP for diagnosing neonatal infection.[23] Infected infants have also been shown to have significantly lower IαIp levels than noninfected infants.[20,21] Nonetheless, the latter studies on IaIp are relatively small and the clinical usefulness of those markers cannot be confirmed at this time. Based on current evidence and the availability of tests in clinical laboratories, most NICUs currently rely on serial measurements of CRP concentration for the identification of infants with LONS.

Chemokines and Cytokines

Chemokines and cytokines have been extensively studied in the past decade. Of this important category of mediators, the proinflammatory cytokine IL-6, the antiinflammatory cytokine IL-10, and chemokines IL-8, IP-10 (10-kDa interferon-γ-inducible protein), and RANTES (regulated upon activation, normal T cell expressed and secreted) have been found to be potentially useful for early diagnosis of LONS and for predicting the severity of infection at the onset of sepsis presentation.[5,10,26]

Proinflammatory mediators are early warning biomarkers because their circulating levels are rapidly and substantially increased after infection.[26,27] Because IL-6 induces the production of CRP in the liver, it is not surprising that its upregulation precedes that of CRP during sepsis.[10] The measurement of IL-6 in conjunction with IL-1 receptor antagonist (IL-1ra) may help predict LONS 2 days before clinical manifestations become evident.[28] IL-1ra has a longer half-life compared with other cytokines, potentially increasing its utility as a sepsis biomarker. IL-6 has a short half-life, and circulating levels decrease precipitously back to the baseline noninfectious state within 24 hours of appropriate treatment.[10] IL-8 (a chemokine) follows a similar time course. This characteristic greatly limits the role of IL-6 and IL-8 as clinically useful biomarkers across all phases of sepsis, although they may have utility in the early presentation before therapy. The quantitation of intracellular IL-8 by treating whole blood samples with detergent to lyse white blood cells may lengthen the window of opportunity for obtaining blood samples and further enhance the diagnostic utility.[29] Other chemokines and cytokines, such as tumor necrosis factor (TNF) α, monokine induced by interferon-γ, monocyte chemoattractant protein (MCP)-1, and growth-related oncogene α, are significantly upregulated during infection and NEC, whereas RANTES is downregulated, in some cases because of concomitant thrombocytopenia.[27] The utility of these diagnostic biomarkers is not as promising as those mentioned earlier.[27]

Another important group of inflammatory mediators is the antiinflammatory cytokines, such as IL-10 and transforming growth factor β, which are important in preventing an exaggerated proinflammatory response during sepsis.[30] An elevated antiinflammatory (IL-10) to proinflammatory (TNF-α) ratio in adult patients with infection has been associated with adverse outcomes.[31] Increased IL-10/TNF-α ratio has also been associated with severe LONS in VLBW infants.[32] In another study, an algorithm using sequential measurements of IL-10, IL-6, and RANTES at clinical presentation was shown to sensitively and reliably predict the development of DIC in severely infected infants.[5] This information is crucial for identifying seriously ill infants who are most in need of urgent treatment and intensive care support and may also assist in counseling of parents at a very early stage of illness. The magnitude and balance (or imbalance) of proinflammatory and antiinflammatory responses are a crucial reflection of the severity of sepsis and may play an important role in predicting morbidity and mortality.[5,31,32] Despite the favorable properties of chemokines and cytokines, assessment using these mediators has not been successfully integrated into routine clinical practice. High-cost, nonautomated, labor-intensive methodology and the lack of on-demand testing in clinical laboratories are major obstacles that have prevented the evolution of cytokines as routine diagnostic tests in the NICU setting.

Cell-surface Antigens

Advances in flow cytometric technology have paved the way to easy detection of cell-surface antigens on circulating inflammatory cells, including neutrophils, lymphocytes, monocytes, and natural killer (NK) cells. Specific cell-surface antigens are expressed in large quantities soon after the target cells are activated by microbial products and bacterial toxins.[3] Many cell-surface antigens have been investigated in relation to neonatal sepsis,[33] and the most promising ones are neutrophil CD64[34–36] and neutrophil/monocyte CD11b.[37–39]

CD64 and CD11b are antigens that are expressed at very low densities on nonactivated white blood cell surfaces. During bacterial and fungal infection or NEC, these antigens are substantially upregulated and their concentrations on the cell surface can be accurately and quantitatively measured by flow cytometry. The authors' findings suggest that neutrophil CD64 is a sensitive biomarker for diagnosis of early-onset

sepsis and LONS,[34–36] and its upregulation significantly precedes that of CRP. Recently, neutrophil CD64 has also been demonstrated to be a good indicator of intra-abdominal sepsis, including NEC, bowel perforation, and peritonitis. As expected, this test is unable to differentiate between systemic infection and intra-abdominal sepsis/inflammation (Lam HS and colleagues, unpublished data, 2010). Neutrophil CD11b has also been suggested to be a sensitive biomarker for early-onset neonatal infection,[37,38] and a subsequent study on daily surveillance showed that neutrophil/monocyte CD11b could reveal evidence of infection up to 3 days before clinical manifestations.[39] Such findings have not been repeated and would require validation by larger studies. Unlike with CD64, the results with CD11b are more variable, and the findings have not been consistent between centers.[34] Expression of CD11b is also influenced by noninfectious conditions such as respiratory distress syndrome.[40] The authors have compared neutrophil CD64 and neutrophil CD11b within the same study and found that CD64 had significantly better utility than CD11b in diagnosing LONS and NEC.[34] Other cell-surface antigens, including NK cell CD69,[41] lymphocyte CD25 and CD45RO,[34] and an elaborate panel of leukocyte surface antigens including CD19, CD33, and CD66b, have also been investigated,[33] but none showed better diagnostic utility than CD64 and CD11b. Advantages of flow cytometry include small blood volume (50 μL whole blood), rapid turnaround time (<4 hours), wide window of opportunity for blood sampling, and ability to perform the test on an ad hoc basis. The disadvantage is that flow cytometry requires skilled technicians to carry out multistep measurements semiautomatically, and flow cytometry is not considered a routine diagnostic evaluation in most NICUs.

Quantitative Polymerase Chain Reaction

The quantitative polymerase chain reaction (qPCR) is a rapid test that can detect bacterial DNA in sterile body fluids, including blood and pleural, peritoneal, and cerebrospinal fluid, and is most desirable when conventional microbiologic methods fail to detect organisms. Molecular techniques such as fluorescence in situ hybridization can substantially reduce the time required to identify organisms isolated in culture.[42] This reduction can be as much as 18 hours for bacterial isolates and 42 hours for yeasts.[42] Directly detecting pathogen DNA is attractive, especially when the target is a fastidious or slow-growing organism. Molecular techniques that focus on identification of a single species of bacteria are not particularly useful in an intensive care setting. An ideal test would encompass a wide variety of pathogens commonly encountered in NICUs. The use of the probe-based gram-specific qPCR for rapid detection and differentiation of gram-negative and gram-positive bloodstream infections has been attempted in recent studies.[4,43] This test has very high specificity and positive predictive value, especially for identification of gram-negative organisms.[4,43] Although the test is not sensitive enough for ruling out sepsis, it is particularly useful for ruling in sepsis because of its high specificity. Thus, a positive test result would strongly indicate the need for a full course of antimicrobial treatment despite negative culture of pathogens.[4] The identification of gram-specific sepsis would serve as a useful guide for prescribing appropriate and effective antibiotics and predicting the virulence of the causative pathogens and severity of infection.[4,43] The major limitations identified in these studies are (1) uncommon organisms not included in the genetic sequence of the primer/probes would escape detection and (2) gram-positive organisms and fungi with elaborate cell wall structures are resilient to digestion and destruction, posing a major problem for DNA extraction.[4] Use of molecular diagnostics for detecting bloodstream infections in neonates has shown some promise and some technical challenges.[44]

Identification of Resistance Genes by Molecular Techniques

The time required for conventional methods to isolate pathogens results in a significant delay of at least several days before vital information on antibiotic sensitivity becomes available. Recently, investigators using microarray-based techniques have demonstrated the possibility of detecting bacterial antibiotic resistance genes within a few hours.[45,46] Studies aiming to identify genes encoding resistance to first-line antibiotics are critically important when choosing empiric therapy in infants with suspected sepsis.

Genomics

Concentrations of cytokines and chemokines within the bloodstream often do not fully reflect the infective or inflammatory process that is ongoing within the patient.[1] Recently, investigators have focused on the possibility of identifying genes that are upregulated in infection. One study measuring whole blood IL-8 and MCP-1 mRNA concentrations suggested that levels of both mRNAs were elevated in infants with perinatal asphyxia, whereas only IL-8 mRNA level was elevated in infants with perinatal infection.[47] The detection of tissue-specific mRNA could potentially be used as a basis for developing disease-specific biomarkers in neonates.

Proteomics

Mass spectrometry–based proteomic profiling technologies, such as surface-enhanced laser desorption/ionization, have been used to identify host response proteins as signatures for diagnosis of a wide variety of pathologic conditions and diseases, such as severe acute respiratory syndrome, intra-amniotic inflammation, and neonatal sepsis.[48] To date, all clinical studies involving acute phase proteins, chemokines, cytokines, and cell-surface antigens selected key mediators or antigens in the infection/inflammatory cascade, using the traditional ''candidate'' approach, and tested their diagnostic utility for LONS. This conventional approach greatly confines and restricts the search for biomarkers to known mediators or proteins of the cascade. In contrast, the proteomics technology with its ''hypothesis-free'' approach can potentially discover novel host response biomarkers for diagnosis of LONS and NEC in preterm infants. The authors recently completed a proteomics project and discovered one known protein and one novel lipoprotein for early and accurate identification of infants with sepsis and NEC.[49] A new proteomics sepsis score derived from these two biomarkers would guide frontline neonatologists whether to 'start' antibiotic treatment at the time of clinical presentation and to 'stop' therapy within 24 hours of commencement. This score ensures patient safety with 100% negative predictive value. It would preclude a significant proportion (>60%) of true non-sepsis cases from receiving antibiotics unnecessarily or for very early withdrawal of treatment. The proteomics technology may further assist in identifying novel biochemical pathways associated with infection. It is a powerful tool for biomarker discovery, and we have demonstrated that the technology can be competently applied to very premature infants. A limitation of proteomics is that proteins with low plasma concentrations, such as chemokines and cytokines, may not be easily detected by this method. In addition, a stringent protocol with elaborate study design is required to ensure that the proteins identified are genuinely representative and specific for the condition investigated.

SUMMARY AND FUTURE CONSIDERATIONS

To date, no single ''ideal'' diagnostic biomarker of LONS has been identified. **Table 1** summarizes the pros and cons of different categories of inflammatory mediators and

Table 1
Advantages and disadvantages of diagnostic biomarkers and tests for LONS

Diagnostic Tests	Diagnostic Utilities	Identification of Specific Pathogens or Conditions	Prediction of Severity and/or Prognosis	Monitoring Progress	Ruling Out vs Ruling in Sepsis	Timing of Specimen Collection	Turnaround Time (h)[b]
Hematologic Tests							
Simple (eg, WCC, differential WCC, I/T ratio, platelets)	Poor	Nonspecific	Fair (neutropenia and DIC in severe sepsis)	Poor	Neither	Any time	4–6
Complex (eg, hematologic scores)	Fair	Nonspecific	?[a]	Poor	Neither	Any time	8–17
Acute Phase Proteins (eg, CRP, SAA)	Good	Nonspecific	Good (higher levels in severe sepsis)	Good	Ruling out	Late	4–6
Chemokines/ Cytokines (eg, IP-10, IL-6, IL-8, IL-10, RANTES)	Very good	Nonspecific	Good (higher levels in severe sepsis)	?[a]	Ruling out	Early	4–6[c]
Leukocyte Surface Antigens (eg, CD64, CD11b)	Very good	Nonspecific	Good (higher levels in severe sepsis)	Fair	Ruling out	Early and late	4[c]
qPCR (eg, gram-specific gene probe)	Very good (gram-negative organisms), fair (gram-positive organisms)	Specific (especially gram-negative organisms)	?[a] (uncertain association with circulating gene copies)	Poor	Ruling in	Early	8–17[c]

Blood Volume (mL)	Measurement				Comments	Routine/ Experimental
	Test Availability	Quantitative (Q)/ Semiquantitative (S-Q)	Automatic Measurement	Low Cost		
0.5–1.0	On demand	Q	Y	Y	DIC	Routine
0.5–1.0	On demand	S-Q	N	?[a]	—	Hematologic score rarely performed
0.5–1.0	On demand	Q	Y	Y	Late biomarker Serial CRP for ruling out sepsis and early stoppage of antibiotics Monitor disease progress	Routine in many NICUs
0.1–0.2 per mediator	Batches	Q	N	N	Early biomarker Predict DIC at the onset Prognostication Multiplex technology for many analytes on small blood volume	Experimental
0.05	On demand	Q or S-Q	N	N	Early biomarker	Selected cases/ experimental
1.0	On demand	Q or S-Q[d]	N	N	Early diagnosis Especially useful in culture-negative cases	Selected cases (especially culture-negative cases)/ experimental

Abbreviations: IP, interferon-γ-inducible protein; N, no; WCC, white cell count, Y, yes.

[a] Uncertain association.

[b] Time from specimen collection to announcement of results.

[c] Tests not routinely available in most clinical laboratories.

[d] Gene copies can be measured.

tests that have been clinically evaluated. The ideal biomarker panel would provide information to facilitate early diagnosis and predict the severity of infection and outcomes at the onset of clinical signs and symptoms. For example, at first suspicion of sepsis, the proteomics sepsis score and early biomarkers, including neutrophil CD64, IL-6, and IL-10, could be used to decide whether to start antimicrobial treatment and also to give forewarning of the severity of sepsis and likelihood of development of DIC.[5,10,32,34,49] At 24 hours, a repeat proteomics sepsis score and neutrophil CD64 could facilitate the decision to discontinue antibiotics in nonsepsis cases.[34,49] Thereafter, serial CRP level measurements may be useful in monitoring the progress or development of late complications.[1,10,11] Molecular diagnostics could be considered for highly suspected cases of sepsis or NEC with negative blood culture[4] or for testing of other sterile body fluids to provide microbiologic information not obtainable by other nonspecific tests.

The clinical research team is primarily responsible for identifying favorable biomarkers and confirming the clinical and laboratory properties of these biomarkers in a typical hospital or NICU setting. One of the main reasons why most favorable biomarkers have not become routine clinical tests is because no automated method of measurement has been developed by the industrial sector. Academic-industry partnership is essential for successful development of new and clinically useful diagnostic biomarkers. Because new molecular and biogenetic technologies are rapidly advancing, nonspecific biomarkers would likely be replaced by more specific tests, which could pinpoint the precise condition (ie, differentiating between septicemia and NEC or focal infections such as pneumonia) and provide vital information on the pathogen and its antibiotic resistance profile within hours of clinical presentation.

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