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EMERGING BIOMARKERS IN NEONATAL SEPSIS

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SUMMARY

Approximately 30-40% of neonatal deaths are associated with bacterial infections, which often progress rapidly and result in high mortality. Because each year almost one million newborns die from infections, mostly in low-income countries, there is a need for a very early, accurate diagnosis of systemic inflammation and sepsis. On the other hand, in the era of multidrug resistance, it is mandatory to avoid unnecessary use of antibiotics to treat noninfected babies and to start the appropriate therapy in those with sepsis. Thus, rapid diagnostic test(s) that differentiate infected from noninfected newborns and surrogate biomarkers predicting outcome have the potential to significantly improve neonatal care. Various emerging biomarkers for neonatal sepsis have been recently proposed. Among these, the most promising biomarkers potentially measurable in clinical practice seem to be lipopolysaccharide-binding protein (LBP), soluble CD14 subtype presepsin (sCD14-ST) and angiopoietin-1 and -2 (ANG-1 and ANG-2, respectively). In the near future, metabolomics could offer a powerful tool to distinguish not only septic from nonseptic newborns, but also to identify without any overlap several clinical conditions associated with infections and inflammation, such as systemic inflammation, systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis and septic shock.

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INTRODUCTION

Neonatal sepsis is a clinical syndrome characterized by signs and symptoms of infection with or without concomitant bacteremia in the first month of life. It encompasses various systemic infections of the newborn, such as septicemia, meningitis, pneumonia, arthritis, osteomyelitis and urinary tract infections (1). Superficial infections like conjunctivitis and oral thrush are not usually included under neonatal sepsis. Neonatal sepsis can be classified into two major categories depending on the onset of symptoms: early-onset sepsis (EOS) and late-onset sepsis (LOS). Neonates with EOS usually present with symptoms within the first 72 hours of life, but in severe cases may be symptomatic at birth. EOS is usually associated with respiratory distress and pneumonia. In LOS, symptoms appear after 72 hours of life. LOS is either caused by nosocomial (hospital-acquired) or community-acquired infection; it is usually associated with septicemia, pneumonia or meningitis (2).

The incidence of EOS in the U.S. and Australasia ranges from 1.5 to 3.5 per 1000 and that of LOS up to 6 per 1000 live births. In Asia, the incidence of neonatal sepsis varies from 7.1 to 38, in Africa from 6.5 to 23, and in South America and the Caribbean from 3.5 to 8.9 per 1000 live births (3). Neonatal sepsis is associated with high morbidity and mortality in newborns, particularly among preterm infants. More than 21% of very-low-birth-weight (VLBW) infants surviving beyond 72 hours have at least one episode of blood culture-confirmed sepsis (4). Moreover, the risk of LOS increases with decreasing birth weight and gestational age (5). In summary, each year almost one million newborns die from infections, mostly in low-income countries (6).

Early warning signs and symptoms are often nonspecific, subtle, inconspicuous and easily confused with noninfective causes, such as apnea of prematurity, transient tachypnea, hypoglycemia, variation in environmental temperature and acute exacerbation of chronic lung disease (7). In most cases, moreover, signs manifest themselves in the absence of a positive culture. By contrast, the clinical course of sepsis and severe sepsis in a neonatal intensive care unit (NICU) can be fulminating, leading to septic shock, disseminated intravascular coagulation and death within hours of onset. In the era of multidrug

resistance, it is mandatory to avoid unnecessary use of antibiotics to treat noninfected babies, as well as to start the appropriate therapy in those with sepsis. Thus, rapid diagnostic tests that differentiate infected from noninfected newborns have the potential to significantly impact neonatal care.

DEFINING SEPSIS BIOMARKERS IN RESEARCH AND CLINICAL PRACTICE

A biomarker is anything that can be measured to extract information about a biological state or process. The National Institutes of Health (NIH) Biomarkers Definitions Working Group has defined a biological marker (biomarker) as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention (8). Biomarkers are the essential tools for the implementation of personalized medicine. Several blood serum markers for sepsis and septic shock have been described and evaluated in the literature. These biomarkers can be classified into four types: diagnostic, monitoring, stratification and surrogate biomarkers (9, 10). Diagnostic biomarkers, including those for screening, recognize the presence or absence of a disease state by a significant increase or decrease in their concentration in plasma. Monitoring biomarkers enables tracking of the evolutionary change and development of a disease, specifically in response to therapeutic interventions, via dynamic variations of the biomarker's levels in plasma. Via specific cutoff levels, stratification biomarkers can be used to sort a patient population into classes of severity based on their biomarker concentration in plasma. Finally, surrogate biomarkers mainly serve as proxy endpoints for death or significant complications; they allow prediction of the outcome of a disease process rather than monitoring of the disease's course or titration of therapy.

CHARACTERISTICS OF AN IDEAL BIOMARKER

Certain universal characteristics have been definitively defined for any biomarker: 1) it should be noninvasive, easily measured, inexpensive and produce rapid results; 2) it should be derived from readily available sources, such as blood or urine; 3) it should have a high sensitivity, allowing early detection, and no overlap in values between diseased patients and healthy controls; 4) it should have a high specificity, being greatly upregulated (or downregulated) specifically in the diseased samples and unaffected by comorbid conditions; 5) its levels should vary rapidly in response to treatment; 6) its changes in concentration should aid in risk stratification and possess prognostic value in terms of real outcomes; and 7) it should be biologically plausible and provide insight into the underlying disease mechanism. Of course, very few biomarkers will meet all of these characteristics.

In newborns, and specifically in preterm newborns, the most readily available source of biomarkers is urine because it can be obtained via normal care for the baby, is easily collected at the bedside and is associated with little or no health risks to the patient. It is an excellent source of biomarkers produced in the kidney, and urinary biomarkers may improve mechanistic insight into specific renal pathologies. Less complex than serum, it is easier to screen for potential biomarkers. Its collection is easy enough, and it can be readily employed in home testing kits. The handling of urine, however, greatly influences the stability of its proteins. Measurements should be made immediately after collection or the urine should be promptly centrifuged, then isolated from sediment, and finally frozen at –80 °C to avoid protein degradation. Results are typically adjusted for urine creatinine to account for differences in urine concentration due to hydration status and medications such as diuretics. Serum and plasma are discouraged as a source of biomarkers in babies. In particular, VLBW and preterm newborns risk blood transfusion after serial blood sampling. Therefore, in neonatology it is unlikely that a biomarker's concentration in plasma can be closely monitored (e.g., every 6 or 12 hours). Plasma biomarkers, however, are more likely to represent a systemic response to disease than an organ response. Even a sensitive biomarker with what experimentally would be considered an excellent specificity of 90% would still yield a false positive rate of 10%, which may be unacceptably high for clinical use as a stand-alone marker (11). As a result, the best approach clinically may be to find multiple biomarkers that can be combined as part of a panel to achieve even higher specificity.

BIOMARKERS FOR SEPSIS DIAGNOSIS AND MANAGEMENT

Despite the considerable number of biomarkers proposed for sepsis, the reliability and validity of most of them have not been tested properly. Therefore, C-reactive protein (CRP) and procalcitonin (PCT) continue to be the most widely used markers in sepsis management. CRP can be considered as a specific but late marker of neonatal infection; changes in its levels are useful to monitor the progress of treatment and for guiding antibiotic therapy. On the other hand, PCT seems to better discriminate systemic inflammatory response syndrome (SIRS) from sepsis, as well as bacterial infections from nonbacterial infections. However, some clinical studies have shown that while PCT is a good diagnostic marker for sepsis, it was not statistically more accurate than CRP (12). More importantly, PCT has a dynamic cutoff range that depends on clinical conditions and setting. Between 48 and 72 hours of life, PCT results should be interpreted with caution; a significant value during that time frame is approximately 1 µg/L. Conversely, after the third day of life, a cutoff of 0.5 µg/L offers good sensitivity and specificity (13). These limitations, together with the need for a very early diagnosis of sepsis, clarify a growing demand for a clinically convenient and reliable marker(s) of neonatal sepsis, severe sepsis and septic shock.

SERUM AMYLOID A PROTEIN AND CYTOKINES

Over the last decade, increasing attention was devolved to serum amyloid A protein (SAA) in neonatal infections and sepsis (14-16). In particular, SAA seems to be an effective marker for the early diagnosis and management of neonatal necrotizing enterocolitis (17, 18), its serum changes being closely related both to the severity of the disease and the effectiveness of therapeutic treatment (19). The term SAA refers to a family of polymorphic apolipoproteins mainly produced by the liver. Currently, the known isoforms are SAA-1α, SAA-2α and SAA-2β. Immediately after its release from the cells, SAA binds with HDL isoform 3 (HDL3) and circulates throughout the system. In newborns, the SAA-1α isoform is predominant, while in older children both SAA-1α and SAA-2α are present. In adults, only the SAA-2α isoform is present (20). Finally, the expression of SAA is increased in response to hypoxia–ischemia of the neonate. The mag-

nitude of the increase in SAA levels in serum significantly correlates with the severity of encephalopathy and is associated with mortality (21). Unfortunately, the most serious limitation to the use of SAA in clinical practice is the lack of standardization of available commercial analytical assays. In recent years, the search for diagnostic tests for sepsis and inflammation in newborns has turned to cytokines and soluble cell surface markers. On the basis of recent findings, cytokine measurement may improve the early identification of newborns with bacterial infections (22). Several cytokines have been widely investigated in neonatal sepsis, especially IL-6, IL-1β, IL-8, IL-12 and TNF-α, granulocyte colony-stimulating factor (G-CSF), soluble TNF receptor (sTNF-R), IL-1 receptor antagonist protein (IL-1RN) and soluble IL-2 receptor (sIL-2R) (23-26). TNF-α is a main initiator of systemic inflammation and, together with IL-6, induces CRP synthesis. Increased concentrations of TNF-α and IL-6 in serum may therefore be detectable before any measurable increase in CRP (27). IL-8 is also likely to be an earlier marker of sepsis because of its involvement in neutrophil bone marrow release and subsequent neutrophil activation and chemotaxis.

SOLUBLE CD14 SUBTYPE PRESEPSIN

Monocyte differentiation antigen CD14 is a multifunctional glycoprotein. It exists either in a glycosylphosphatidylinositol (GPI)-anchored membrane form, expressed on the surface of various cells, including monocytes, macrophages, neutrophils, etc., or in a soluble form (sCD14). Upon binding of the lipopolysaccharide (LPS)/lipopolysaccharide-binding (LBP) complex, CD14 activates the Toll-like receptor 4 (TLR4)-specific proinflammatory signaling cascade, thereby starting the inflammatory reaction of the host against infectious agents. The complex LPS/LBP/CD14 is released into the circulation by shedding of CD14 from the cell membrane, yielding sCD14. In addition, sCD14 is also directly secreted by hepatocytes, leading to the conclusion that sCD14 should be considered as a minor acute-phase protein. Circulating plasma proteases cleave sCD14, generating a truncated form of 64 amino acid residues named sCD14 subtype (sCD14-ST), or presepsin (28). By interacting directly with T and B cells, sCD14-ST is a regulatory factor capable of modulating cellular and humoral immune responses (29). Its levels in plasma specifically increase during sepsis and, less intensively, during SIRS, with magnitude correlated with the phagocytosis process and cleavage with lysosomal enzymes from bacteria and microorganisms. Moreover, an increased sCD14-ST concentration in serum has been correlated with IL-8 levels and poor outcomes for patients with sepsis. However, sCD14-ST reduces the mortality rate caused by endotoxic shock and the severity of Gram-negative bacterial infections. Healthy neonates have reduced sCD14 levels in cord blood or plasma on the first day of life compared with healthy adults. Additionally, neonates did not respond with excessive sCD14 release during infection, as has been shown for adults with levels of up to 20 mg/L in plasma in polytrauma or sepsis (30). The concentration of sCD14 at a postnatal age of approximately 2 weeks is strictly comparable to levels in adults: 1.73 ± 0.83 mg/L versus 1.4 ± 0.29 mg/L, respectively (31). Very recently, a new commercially available chemiluminescent one-step ELISA test was optimized and validated for the routine measurement of plasma sCD14-ST (32). A preliminary cutoff value of 415 µg/L permits a sensitivity of 80.1% and a specificity of 81% (33).

LIPOPOLYSACCHARIDE-BINDING PROTEIN

LBP is a 50-kDa polypeptide mainly synthesized in the liver and released into the bloodstream as a 58- to 60-kDa glycoprotein after glycosylation. Human LBP consists of 452 amino acid residues in the mature protein, containing the typical 25-amino-acid signal sequence characterizing secreted proteins. It belongs to the acute-phase proteins and is primarily synthesized by hepatocytes, but also by epithelial and muscle cells (34). LBP belongs to the lipid transfer/LBP family comprising bactericidal permeability-increasing protein (BPI), cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP). LBP binds and recruits the endotoxin to the surface of Gram-negative bacteria very early and during infections; it is the main plasma protein responsible for transporting endotoxin to immune effector cells bearing CD14 on their surface (35). LBP also recognizes a number of microbe-associated molecular patterns (MAMPs) commonly associated with Gram-positive bacteria (lipoteichoic acids and peptidoglycan), mycobacteria (lipoproteins and lipomannans), mycoplasms (lipopeptides) and spirochetes (glycolipids and lipoproteins). LBP has a dual role in interactions with LPS: at low concentrations it enhances LPS signaling by extracting it from bacterial membranes and transferring LPS monomers to CD14 (150 molecules per minute), while at high concentrations it inhibits LPS signaling by shuttling it to serum lipoproteins and forming LPS aggregates (36).

Increased secretion of LBP stimulated by LPS is found in the serum of patients with sepsis, and therefore LBP serves as an inhibitor of the excessive response to LPS. LBP levels in plasma have been investigated in various human populations: in healthy subjects, the LBP concentration in plasma is 2-10 mg/L (37), while very high levels exceeding 100 mg/L have been observed in patients with sepsis, SIRS, abdominal infections, ulcerative colitis and many other diseases. LBP can be routinely measured in clinical laboratories by an automated solid-phase enzyme-labeled chemiluminescent immunometric assay (Immulite® 2000; Siemens Healthcare Diagnostics). At the pediatric and neonatal age, LBP has been found to be a specific and sensitive marker capable of discriminating between SIRS and bacterial infection (38). Especially in preterm babies and in children with cancer at the onset of febrile neutropenia (FN), increased LBP concentration in serum is an early marker of bacterial infections. In healthy full-term newborns, LBP levels in serum are closely comparable to those in adults. During EOS, a significant increase in serum LBP has been demonstrated compared with that in healthy full-term newborns (39). The LBP cutoff value identifying septic syndrome has been reported for newborns (> 7.4 mg/L) and is nearly identical to that for adults (40).

ANGIOPOIETIN-1 AND -2

Endothelial barrier disruption plays a key role in the pathogenesis of sepsis and septic shock, making it an attractive therapeutic target (41). Thus, biomarkers reflecting endothelial cell state may be useful for tracking sepsis (42). Angiopoietin-1 (ANG-1) and -2 (ANG-2) are angiogenic factors belonging to the family of growth factors that have been studied mainly in proliferative diseases characterized by neoangiogenesis, such as cancer (43). ANG-1 is secreted mainly by smooth muscle cells, pericytes and astrocytes. It primarily plays a role in maturation of newly formed blood vessels and stabilization of

existing vasculature, but also contributes to initiation of neovascularization. ANG-1 and ANG-2 are antagonists that trigger endothelial cell activation, involving the most important intracellular pathways (nuclear factor 1 B type [NF1-B] for inflammation, Rho-associated protein kinase (ROCK) for interendothelial cell contacts and the phosphatidylinositol 3-kinase [PI3K]/Akt pathway for cell survival). Both ANG-1 and ANG-2 bind to the endothelial tyrosine-protein kinase receptor TIE-2 (44). TIE-2 is believed to play an important role in stabilization/destabilization of endothelial integrity, as well as in angiogenesis by involving processes such as vessel integrity, vascular permeability and the regulation of inflammation (45). ANG-1 serves as a TIE-2 agonist, whereas ANG-2 was initially thought to serve primarily as a functional antagonist (46). ANG-1 closely interacts with vascular epithelial growth factor (VEGF) in initiating angiogenesis. The two proteins act in a coordinated way during embryogenesis for vascular development (47). However, VEGF is more involved in the initial formation of vasculature, whereas ANG-1 is integral in vascular remodeling and maturation into functional blood vessels (48). Both ANG-1 and VEGF stimulate expression of inflammatory cytokines before angiogenesis. Angiopoietins can directly stimulate both endothelial cells and neutrophils for an overall proinflammatory and proangiogenic response. The chemotactic effects of ANG-1 on neutrophils are regulated by PI3K activation (49).

Several investigations have demonstrated the importance of the ANG/TIE-2 system in systemic inflammatory disorders. In critically ill patients, the release of ANG-2 directly reflects vascular barrier breakdown. In addition, ANG-2 serves as a marker to discriminate between sepsis and severe sepsis ($P < 0.05$), with variations closely similar to those of TNF-α and IL-6, as recently reported (50). In individuals with FN, the relative concentrations of ANG-1 and ANG-2 were found to be different in patients developing noncomplicated sepsis than in those developing septic shock. Also, the evaluation of these two proteins within the first 48 hours after FN was shown to be a promising tool to discriminate high-risk patients with FN before the development of any signs and symptoms of septic shock (51). Further studies demonstrated that circulating ANG-2 levels correlated with the APACHE and SOFA scoring systems, as well as with the 28-day mortality reflecting disease severity and prognosis (52-54). In children with severe bacterial infection, circulating low ANG-1 and higher ANG-2 concentrations are associated with an unfavorable outcome (55). Therefore, angiopoietins could be considered useful markers for the early identification of patients at risk of a poor outcome, being directly linked to the endothelial damage/dysregulation occurring in severe bacterial infection.

The predictive power of ANG-1 and ANG-2 as prognostic markers in the course of sepsis was recently confirmed (56); in 70 patients with sepsis, both ANG-1 and ANG-2 levels correlated strongly with 28-day mortality. In addition, ANG-2 levels correlated with disease severity, as reflected by markers of organ damage and clinical sepsis scores. These studies suggest that ANG-1 and ANG-2 seem to be of interest as prognostic sepsis biomarkers, reflecting the direct status of the endothelium, which correlates with disease severity and outcome. At admission to the NICU, ANG-1 levels might predict outcome, whereas ANG-2 might be of interest for monitoring newborns with sepsis, showing similar patterns as TNF-α and IL-6 (57).

METABOLOMICS IN NEONATAL SEPSIS

Due to multiorgan dysfunction, neonatal sepsis can result in large changes in the circulating metabolome. For this reason, analysis of the whole metabolic profile using the metabolomics approach offers an attractive modern methodology for fast and comprehensive determination of multiple circulating metabolites and for defining the metabolic phenotype of sepsis. Metabolomics technologies consist of holistic, rather than reductionist, approaches to the molecules that make up a cell, tissue or organism (e.g., genes, transcripts, proteins and metabolites). In this context, metabolomics may aid in the early diagnosis and classification of a pathological condition developing during the perinatal period, as well as in the prediction of its evolution. By providing access to a portion of biomolecular space not covered by other profiling approaches (e.g., proteomics and genomics), metabolomics offers unique insights into biological small molecule regulation and signaling. Metabolomics analysis of biofluids and tissues has been successfully used in the fields of physiology, diagnostics, functional genomics, pharmacology, toxicology and nutrition (58-60). Recent studies have evaluated how physiological variables or pathological conditions can affect metabolomic profiles.

Lindon and Nicholson defined metabolomics as "a global holistic overview of the personal metabolic status", or, in other words, "a snapshot of the chemical fingerprints that specific cellular processes leave behind" (61, 62). Metabolomics can also be defined as the study of the complete set of metabolites and/or low-molecular-weight intermediates, which is context-dependent, varying according to the physiology, developmental or pathological state of the cell, tissue, organ or organism. Endogenous metabolites in biofluids can describe a cellular phenotype. At the moment, one limitation is the fact that the human metabolome is not well characterized, but with the help of the human metabolome project, started in 2005 with the support of the Canadian government, it will be in the near future. The metabolomics technique is applicable to any nucleus possessing a spin and it is highly reproducible, relatively rapid and generally requires no sample extraction. It includes nuclear magnetic resonance (NMR) and isotope dilution liquid chromatography/mass spectrometry (ID-LC/MS). The NMR technique is characterized by the ability to explore the whole metabolic profile and it does not require any sample extraction or modification. However, it has low sensitivity, difficulties recognizing coresonant metabolites, and pH is a parameter that needs to be controlled to avoid shifting of metabolite peaks and other physical/chemical interactions. ID-LC/MS is an analytical technique used to determine masses of particles according to their mass/charge ratio, with the aim of looking at molecules' chemical structure. Compared with NMR, ID-LC/MS is a more accurate technique, but it always requires a sample extraction and preparation. Analysis with these techniques produces series of spectra composed of complex datasets resulting from the measurements of a discrete number of metabolites in several samples. Nowadays, a serious challenge in spectroscopy lies in the technical capacity to generate data and the human capacity to interpret and integrate them. It is important to note that a complex system such as biofluids is characterized by many hidden relationships, and data mining techniques are required to find these relationships in complex datasets. The question as to what type of biological sample should be used in metabolomics depends on a

study's objective. Urine and blood plasma or serum are the most commonly used samples for metabolomic studies. They are obtained essentially noninvasively and can be easily used for clinical purposes. The number of metabolites detected by metabolomic analysis depends on the biological sample examined, and can reach up to several thousand (63). This is because different tissues/biofluids have different functions or play various metabolic roles.

To our knowledge, very few experimental and clinical investigations on neonatal sepsis using the metabolomics approach have been published. In a recent paper, Izquierdo-Garcia et al. (64) collected and analyzed bronchoalveolar lavage fluid (BALF), lung tissue and serum samples from 28 male Sprague-Dawley rats, 14 of which underwent sepsis due to cecal ligation and puncture and 14 controls. [^{1}H]-NMR-based metabolomics absolutely discriminated between the two groups, and showed significant changes on metabolites describing the differences. In addition, the metabolites were used to build a predictive model for the diagnosis of sepsis, which reached 100% sensitivity and specificity. In 2010, Liu et al. (65) used ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS) on plasma samples investigating thermally injured and/or septic rats. The metabolomics approach was able to distinguish between rats suffering from septicemia due to thermal injury and a group of controls without sepsis. In addition, using a mathematical model describing the sepsis, the authors discovered nine metabolites involved in oxidative stress and tissue damage.

The number of metabolomics applications and techniques seems to be growing exponentially, and it is already clear that this strategy will have a significant impact on the discovery of unique metabolic signatures that may be related to clinical sepsis. Based on this technology, it will also be possible to predict the outcome of sepsis. Metabolomics considering latent hidden variables may help to develop a therapy for use before intervention.

DISCLOSURES

The authors state no conflicts of interest.

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