The importance of biomarkers in neonatology

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SUMMARY

Despite a 35% decline in the mortality rate for infants aged <5 years over the past two decades, every year nearly 40% of all deaths in this age group occur in the neonatal period, defined as the first 28 days of life. New knowledge on molecular and biochemical pathways in neonatal diseases will lead to the discovery of new candidate biomarkers potentially useful in clinical practice. In the era of personalized medicine, biomarkers may play a strategic role in accelerating the decline in neonatal mortality by assessing the risk of developing neonatal diseases, by implementing tailored therapeutic treatment, and by predicting the clinical outcome. However, there is an urgent need to reduce the gap in translating newly acquired knowledge from bench to bedside. Traditional and candidate biomarkers for neonatal sepsis and necrotizing enterocolitis will be discussed in this review, such as C-reactive protein (CRP), procalcitonin (PCT), serum amyloid A (SAA), soluble form of CD14 subtype presepsin (sCD14-ST), lipopolysaccharide binding protein (LBP), angiopoietins (Ang)-1 and -2, soluble form of triggering receptor expressed on myeloid cells (sTREM-1), soluble form of urokinase-type plasminogen activator receptor (suPAR), platelet-activating factor (PAF) and calprotectin. New frontiers in managing critically ill newborns may be opened by metabolomics, a diagnostic tool based on the recognition of metabolites contained in biological fluids. Metabolomics represents the passage from a descriptive science to a predictive science, having the potential to translate benchtop research to real clinical benefits.

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1. Introduction

Neonatal mortality is increasingly important because the proportion of deaths of infants aged <5 years occurring during the neonatal period is increasing as the mortality for infants of this age group declines.1 Despite a 35% decline in the mortality rate of infants aged <5 years over the past two decades, every year nearly 40% of all deaths of this age group occur in the first 28 days of life.2 The neonatal period. In 2010, neonatal mortality was estimated to be 23 deaths per 1000 live births worldwide, equal to 3.06 million babies dying in the neonatal period.3 Only 1% of neonatal deaths occur in high-income countries, corresponding to an average neonatal mortality rate of 4 per 1000 live births. Finally, 75% of all neonatal deaths occur within the first seven days of life and almost 33% of these babies die during the first 24 h of life.4 Infections (pneumonia, diarrhea, and tetanus), asphyxia, and preterm birth, together account for nearly 80% of these deaths.5 Deaths caused by preterm birth, asphyxia, and congenital defects occur predominantly during the first week of life, whereas infections are the major cause of neonatal deaths thereafter. A relevant indirect cause of neonatal mortality is low- and very low birth weight (LBW and VLBW, respectively). Although globally only 16% of newborns have LBW, 60–80% of neonatal deaths occur in LBW infants. LBW is due to short gestation (preterm birth), intrauterine growth restriction (IUGR), or both. Globally, almost one-third of neonatal deaths are directly attributable to preterm birth. Consequently, a baby born with LBW, especially if preterm, is at much greater risk of dying or of becoming sick than other newborns.

A great deal of effort in reducing neonatal mortality rate is focused on looking for new biochemical markers able to predict early the risk of developing neonatal acute diseases and to accurately monitor the course of the disease in acute critically ill newborns. Significant challenges arise in the discovery of clinically useful biomarkers: new knowledge on molecular and biochemical pathways in human diseases calls for renewed efforts in searching for biomarkers closely related to specific pathological processes or to a disease state with increasing sensitivity and specificity. In the era of personalized medicine, biomarkers are the essential tools for...
the implementation of personalized care. Ushered in by the remarkable genomic, proteomic and metabolomic advances in our understanding of health and disease, personalized medicine promises a more precise determination of disease predisposition, diagnosis and prognosis, earlier preventive and therapeutic interventions, a more efficient drug development process, and a safer and more fiscally responsive approach to medicine. Neonatal care could substantially benefit from the application of emerging molecular technologies, especially to improve disease-specific therapies and monitor responses to drug administration.

2. Biomarkers

In recent decades biomarkers have become essential in diagnosing disease and assessing patients’ responses to therapy. In fact, simple, inexpensive, reliable, and rapidly obtainable measures of a disease process may have great utility both in clinical care and clinical trials. These types of measures, referred to as biomarkers and surrogate endpoints, may be useful for diagnosis, prognosis, therapy, and drug development. Critically ill newborns provide examples of many important issues related to the development and use of these measures and may represent unique challenges to their acceptance.

In 1999, the US National Institutes of Health (NIH) together with the US Food and Drug Administration (FDA) cosponsored a conference on ‘Biomarkers and Surrogate Endpoints: Advancing Clinical Research and Applications.’ Two years later, the NIH working group on biomarker definition summarized the concepts of biomarkers and surrogates in three key points: (i) a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention; (ii) a clinical endpoint is a characteristic or variable that reflects how a patient feels, functions, or survives; (iii) a surrogate endpoint is a biomarker that is intended to substitute for a clinical endpoint.

Similarly, the International Program on Chemical Safety, led by the World Health Organization (WHO) together with the United Nations and the International Labor Organization, has defined a biomarker as ‘any substance, structure, or process that can be measured in the body or its products, and influences or predicts the incidence of outcome or disease.’ More complex definitions have been subsequently proposed, and recently they have been partially reviewed. However, the NIH definitions represent the best, concise summary of the basic concepts.

Biomarkers are considered surrogate endpoints when used as outcomes in clinical trials: in that case, biomarkers act as surrogates or substitutes for clinically meaningful endpoints. However, not all biomarkers can be used as outcomes in clinical trials, nor are they all intended to be. Robust scientific evidence (e.g. epidemiological, therapeutic, and/or pathophysiological) supporting the capacity of a biomarker to consistently and accurately predict clinical outcome are mandatory before considering the biomarker as a surrogate endpoint. In this sense, a surrogate endpoint is a biomarker that can be trusted to serve as a stand-in for, but not as a replacement of, a clinical endpoint. Consequently, biomarkers play a strategic role for the future of health care, having many potential applications in research and medicine. The advent of large-scale genomic, proteomic, metabolomic, and advanced imaging technologies has rapidly changed the environment in which biomarkers are identified and measured. By providing a measure of a biological state, they can estimate either normal biological processes or pathogenic processes. At least four different classes of biomarkers can be categorized, on the basis of their clinical role: screening, staging, antecedent, and prognostic biomarkers. Screening biomarkers are indicators of disease trait: genetic testing and familial screening in inherited genetic diseases as well as tests investigating systemic or local injuries due to environmental exposures may be considered good examples. Staging biomarkers reflect the disease state and the rate of the disease progression, such as alteration in C-reactive protein (CRP) serum concentration during bacterial infection. Antecedent biomarkers identify people at high risk of developing disease: for example, mannosese-binding lectin (MBL) is considered a risk factor for systemic inflammation and sepsis. Finally, prognostic biomarkers enable prediction of outcome during the follow-up of patients with multiple risk factors: the independent association between thrombopoietin and the severity of infection during systemic inflammation and sepsis may be considered an excellent example. Biomarkers can indicate whether a drug is having an effect in an individual and whether side-effects can be anticipated. Biomarkers also have many potential applications in the development of drugs.

3. Research steps in biomarker development

The development of a biomarker is a process starting with biomarker discovery; it is followed by rigorous evaluation of classification accuracy, and ends with the evaluation of the impact of the biomarker on clinical outcomes. Multiple studies should be involved in each stage. The process can be summarized by categorizing it into three broad phases: the biomarker discovery, the evaluation of biomarker classification performance, and the impact of using biomarkers in clinical care. Researchers and manufacturers claim patents on their biomarker discovery, even if these patents have generated controversy over whether they hinder the practice of medicine and research by covering not just the actual test but also the use of the biomarker generally in making diagnoses and discovering new applications. Biomarker discovery consists of the identification of a candidate biomarker for the disease of interest, involving two complementary approaches: the ‘knowledge-based’ (deductive method) and the ‘unbiased’ (inductive strategy). The knowledge-based strategy consists of a direct understanding of the underlying processes, such as the activation of molecular pathways during systemic inflammation and the effects on the disease of such activated factors. This strategy may be oriented to improve existing biomarkers to enhance their performance or to develop new assays for new candidate markers. The unbiased approach involves examining tens of thousands of molecules using current technological advances to characterize the biomolecular signature of a stage of the disease. The second phase is strategic to establish whether a candidate biomarker can discriminate between diseased and non-diseased patients, also exploring covariates associated with the biomarker. During this phase, it is of great value to determine whether the biomarker precedes current standard methods for diagnosing the disease. The third phase investigates the impact of biomarker in clinical care, determining cost-effectiveness and improvement in outcomes. Each phase requires unique statistical considerations and tailored study design to accurately evaluate research objectives.

4. Biomarker specifications

The most important properties of a biomarker include sensitivity, specificity, calibration, discrimination, and reclassification. The sensitivity and specificity of a biomarker go hand in hand; they represent the accuracy of a biomarker. Sensitivity is the proportion of truly affected cases (patients) in a screened population who are identified as being diseased by the test, and is a measure of the probability of correctly diagnosing a condition. Specificity is the proportion of truly non-diseased persons who are identified as such by the screening test. The receiver operating characteristic (ROC) curve is a binary classification test, based on the sensitivity
and specificity of a biomarker at certain cut-off values. ROC curves are often used to determine the clinical diagnostic value of a marker.\textsuperscript{21} An area under the ROC curve (AUC) of $\geq 0.75$ is commonly considered a good biomarker, an AUC of 0.90 is considered excellent, and an AUC of 1.0 represents a perfect (ideal) biomarker. An AUC of 0.5 is a result that is no better than expected (classification).\textsuperscript{22} Apart from their role, the effective applicability of biomarkers in a clinical setting involves high sensitivity and specificity of an assay or investigation, furnishing the result with an unequivocal message of either high positive predictive value (confirmation of disease) or negative predictive value (exclusion).\textsuperscript{23} Biomarkers should fulfill strategic attributes depending on the disease: in acute clinical settings, a biomarker should allow a very early detection of the disease within a critical time-window; importantly, the close correlation between biomarker release and disease extent together with the speed of the assay are critical.\textsuperscript{24} In chronic diseases, narrow intra-individual variation in biomarker concentration should accurately reflect disease monitoring, outcome, and therapy effectiveness.

Calibration refers to the agreement between the predicted probability of an outcome and the actual probability when measured in a population. Discrimination refers to the ability of a biomarker to distinguish those with a disease or event from those without. Reclassification refers to the ability of a biomarker measurement to move the probability of an outcome beyond a threshold that leads to a different diagnosis, prediction of outcome, or clinical decision than would have been made based on prior information. The synthesis of these measures is complex since biomarkers can be excellent for some purposes and mediocre for others, thereby complicating their use for decision-making. For example, if a biomarker has high sensitivity but low specificity, most of the truly at-risk cases will be correctly identified, but many of the not-at-risk cases will also be identified as at-risk.

Each candidate surrogate biomarker should be evaluated beforehand by assessing its relevance, qualification, and validity. Relevance is the biomarker’s ability to appropriately provide clinically relevant information on questions of interest to the public, healthcare providers, or health policy officials. Qualification is a measure of the use of a biomarker in a specific context. That context may be selecting or deselecting people for a clinical trial, monitoring drug-induced toxicity, or some other purpose. Validity is the need to characterize a biomarker’s effectiveness or utility as a surrogate endpoint. However, clinical validity is more than just evidence of biomarker–disease association; it must also include the evaluation of clinical test performance (sensitivity, specificity, positive and negative predictive value and other test parameters) and its impact on health outcomes. The standardized approach for validation includes comparison with a ‘gold standard’ of practice of an adequate sample of patients within a defined spectrum of a certain condition. A sound validation process typically requires two sets of tests: an initial preliminary training set and a validation or test set.\textsuperscript{25} Validity should also be referred for analytic validation, which is a measure of how well an assay detects or quantifies a constituent under various conditions. Validation thus would require demonstration of the performance characteristics of an assay. Analytic validation is necessary but generally not sufficient for a biomarker: it requires robust analytical quality specifications (reproducibility, accuracy, linearity, etc.) and standardization to facilitate the comparison of results across laboratories. Validation also requires study of variability between users and between laboratories. Finally, validation requires an understanding of the potential for drugs or other conditions to interfere with results. By contrast, qualification requires context-specific measurement of the performance of the biomarker in relation to an outcome or outcomes of interest.

5. Searching new candidate biomarkers: from bench to bedside

There is clearly the need for more and better innovation in clinical laboratory tests; however, the road that brings biomarkers from bench to bedside is notoriously bumpy.\textsuperscript{26} Currently, there is a gap in translational research between the identification of a biomarker associated with a particular disease and demonstrating that newborns tested for this biomarker have better outcomes than those who are not. On the one hand, the number of candidates for translational research is continuously increasing, whereas on the other hand the likelihood of success is small.\textsuperscript{27} Evidence-based decisions require information regarding the diagnostic performance, clinical impact, analytical quality specifications, organizational impact and cost-effectiveness of a candidate surrogate biomarker.\textsuperscript{28} In fact, identifying a biomarker—disease association is necessary, but not sufficient, for effective clinical performance. Evidence for a particular test should include: (i) biomarker–disease association; (ii) technical performance (analytical validity); (iii) assessment of clinical validity and test purpose; (iv) assessment of clinical utility and impact; (v) use of the test in different clinical situations (tied to care pathways) and for particular purposes; (vi) any specific circumstances where use of the test has been reviewed and is regarded as unjustified. Unfortunately, there are no systematic processes and platforms for generating clinical data (akin to Phase III pharmaceutical studies) to inform test evaluation, and no agreement about whose responsibility it should be to provide the resources for, or to carry out, such studies. Furthermore, there is no worldwide consensus about the standards required and no organizations have a specific responsibility for systematically analyzing and documenting results from studies of diagnostics and biomarkers.\textsuperscript{29} The conspicuous gap in translational research is often related with the choice of the gold standard for discriminating true positives from true negatives. A paradigmatic example may be the clinical validation of the next-generation biomarkers for kidney disease. The most investigated biomarker during the past decade was neutrophil gelatinase-associated lipocalin (NGAL) for assessing acute kidney injury (AKI) in adults, children, and newborns. In all clinical studies, patients have been identified as AKI and non-AKI on the basis of serum creatinine, a functional parameter of glomerular filtration and not a clinical endpoint. Unfortunately, in non-steady-state conditions such as AKI, serum creatinine is a retrospective, insensitive and even deceptive measure of kidney injury; moreover, serum creatinine does not identify the cell type that is acutely injured even though this localization determines the natural history of the disease and its response to therapy. Therefore, it is obvious that results may be altered by inclusion of AKI patients in the group of non-AKI patients.\textsuperscript{30} As previously stated by Davis and colleagues in 2003, ‘a large gulf remains between what we know and what we practice’.\textsuperscript{31}

6. New frontiers in biomarkers research for neonatal sepsis

Every year, almost one million newborns die from infections, mostly in low-income countries.\textsuperscript{32} In the USA, the overall incidence of early-onset neonatal sepsis ranges between 0.76 and 0.77 cases per 1000 live births, with black preterm infants showing the highest incidence (5.14 cases per 1000 live births) and a case fatality of 24.4%.\textsuperscript{33} Sepsis originates from an abrupt evolution of infections, but it is supported by a cytokine-mediated condition consisting of immune, inflammatory, and coagulation homeostasis impairment.\textsuperscript{34} The balance between the pro–anti-inflammatory factors involved in the course of the diseases (cytokines and secondary mediators) basically influences the evolution of the disease. The clinical course of neonatal sepsis may suddenly
progress toward shock, disseminated intravascular coagulation (DIC), and death within a few hours from the onset of the disease.\textsuperscript{35} Therefore, the most relevant risk factors for mortality are septic shock and the subsequent multiple organ dysfunction syndrome (MODS). As many as 21\% of VLBW babies suffer from one or more episodes of blood-culture-proven late-onset infection before discharge from the neonatal intensive care unit (NICU).\textsuperscript{36,37} Additional causes of death are long-term complications, e.g. chronic lung disease, neonocognitive disabilities together with neurological sequelae, growth retardation, etc.\textsuperscript{38} The most serious problem with the diagnosis of septic newborns is the difficulty of early recognition of the developing systemic inflammation and sepsis related both to the non-specificity of clinical features and to the very frequent absence of a positive microbiological culture. Early warning signs and symptoms are often non-specific, subtle, and inconspicuous and can be easily confused with apnoea of prematurity, transient tachypnea, hypoglycemia, variation in environmental temperature and acute exacerbation of chronic lung disease. Over the last decade, progress has been made in the search for new molecular microbiological tests\textsuperscript{39} and biochemical markers\textsuperscript{40,46} for the early, accurate identification and management of neonatal sepsis; however, an optimal biomarker demonstrating a high sensitivity (close to 100\%) in order to avoid the risk of missing genuine infected cases, together with a high negative predictive value in order to rule out non-infected babies, has never been identified. There are very few biomarkers for neonatal sepsis with a clearly defined cut-off value for distinguishing between infected and non-infected newborns in the early stage of the disease.\textsuperscript{41} In the NICU, CRP and procalcitonin (PCT) continue to be the most widely used markers for sepsis management. CRP is a very specific, but not very sensitive biomarker of bacterial infection; however, the evolution of severe sepsis and septic shock may not be closely correlated with changes in serum CRP concentration. Serial CRP measurements are clinically useful in monitoring the response to therapeutic treatment in neonatal infections, to determine the length of antibiotic therapy, and to assess possible complications.\textsuperscript{42}

PCT has the advantage of quickly increasing within 4 h from the start of the innate immune cascade, reaching the peak within 6–8 h, and thus it is widely used for monitoring sepsis in newborns.\textsuperscript{43} Unfortunately, PCT has dynamic reference intervals and cut-off ranges, depending on gestational age, postnatal age, clinical conditions and settings.\textsuperscript{44} Between 48 and 72 h of life, PCT results should be interpreted with caution, being significant values of about 1 \(\mu\)g/L while after the third day of life, a cut-off of 0.5 \(\mu\)g/L offers good sensitivity and specificity.\textsuperscript{45} Serum amyloid A (SAA; a term including a family of polymorphic apolipoproteins) rises more quickly than CRP and has a better prognostic value during the first 24 h after sepsis onset.\textsuperscript{46} The lack of standardization of available commercial analytical assays seriously limits the introduction of SAA in clinical practice. The search for diagnostic tests for sepsis and inflammation in newborns has turned in recent years to cytokines and chemokines, especially interleukin (IL-)6, -8, and -10, interferon (IFN)-\(\gamma\), and tumor-necrosis factor (TNF)-\(\alpha\). Their serum concentrations change in the course of systemic inflammation and sepsis, depending on their own pro- or anti-inflammatory role.\textsuperscript{47} IL-6 is the most investigated cytokine in neonatal infections and sepsis: despite its very high sensitivity (nearly 100\% in early-onset sepsis), the clinical usefulness is reduced by its very short half-life, leading to a rapid decrease in sensitivity after the start of therapy (67\% after 24 h and 58\% after 48 h). Therefore, there is a relatively narrow window of opportunity for using IL-6 in neonatal sepsis and inflammation.\textsuperscript{48}

Two acute-phase proteins, soluble CD14 subtype presepsin (sCD14-ST) and lipopolysaccharide binding protein (LBP), have been previously investigated in several clinical studies; each of these studies had various limitations consisting of inaccurate analytical methods, inadequate population size, incorrect data analysis, etc. The recent availability of standardized commercial kits, easily adaptable on automated analytical platforms, has aroused interest in these markers, because automation in analytical methods consistently reduce the turnaround time (TAT) of sCD14-ST and LBP up to values closely comparable with those of CRP and PCT. In particular, sCD14-ST presepsin can be easily measured by a chemiluminescent one-step enzyme-linked immunosorbent assay (ELISA)\textsuperscript{49}; a cut-off value of 415 \(\mu\)g/L permits sensitivity of 80.1\% and specificity of 81\%.\textsuperscript{50} LBP can be measured by an automated solid-phase enzyme-labeled chemiluminescent immunometric assay\textsuperscript{51}; the reference value ranges 2–10 mg/L and a cut-off value of 7.4 mg/L discriminates septic syndrome in newborns and adults.\textsuperscript{52,53} Therefore, routine measurement of sCD14-ST and LBP may represent a challenge in searching for reliable markers for the very early diagnosis, classification into class of severity, and prediction of complications and death in septic newborns.\textsuperscript{54}

Fundamental research driven by scientists’ interest in discovering pathways involved in innate immunity and in endothelial barrier disruption have led to the evaluation of a new generation of biomarkers for sepsis.\textsuperscript{55} The evolution of severe sepsis toward septic shock comes with serious alterations in the intravascular function of the microcirculation which, in turn, play a strategic role in the pathogenesis of MODS.\textsuperscript{56} Thus, biomarkers reflecting endothelial cells state might be useful for tracking sepsis.\textsuperscript{57} Angiopoietins (Ang)-1 and -2 and the tyrosine kinase receptor Tie2 are crucial regulators of endothelial cells;\textsuperscript{58} binding of Ang-1 to Tie2 maintains the quiescent resting state of the endothelium and reduces the vascular permeability in response to inflammation.\textsuperscript{59} Ang-1 has mainly anti-inflammatory properties by inhibiting activation of nuclear factor \(\kappa\)B (NF\(\kappa\)B) and by reducing vascular permeability in response to inflammatory stimuli.\textsuperscript{60} On the other hand, Ang-2 appears to have pro-inflammatory properties by increasing vascular leakage.\textsuperscript{61} Ang-2 inhibits binding of Ang-1 to Tie2, resulting in vessel destabilization.\textsuperscript{62} Angiopoietins can directly stimulate both endothelial cells and neutrophils for an overall pro-inflammatory and pro-angiogenic response. Briefly, Ang-1 mRNA is mainly expressed in peri-endothelial cells, whereas Ang-2 mRNA is selectively expressed in endothelial cells. Ang-1 promotes stability of blood vessels and Ang-2 promotes vascular destabilization and permeability.\textsuperscript{53} Plasma Ang-2 levels have been found significantly increased in children with systemic inflammatory response syndrome (SIRS), sepsis, and septic shock when compared with healthy children whereas in children with septic shock, plasma Ang-1 levels were significantly lower than those in critically ill children with either SIRS or sepsis.\textsuperscript{64} In children with severe bacterial infection, circulating low Ang-1 and higher Ang-2 concentrations are associated with an unfavorable outcome.\textsuperscript{65} Recently, it was found that Ang-2 significantly increases in septic shock; this increase is related to unfavorable outcome.\textsuperscript{66} Moreover, a circulating factor may exist in patients with septic shock stimulating gene expression and subsequent release of Ang-2 from monocytes and probably by other cell types. On the basis of current knowledge and of the experimental findings reported in the literature, we can argue that Ang-1 may predict outcome at the admission time in NICU, whereas Ang-2 might be of interest for monitoring septic newborns.

The triggering receptor expressed on the myeloid cell (TREM) family is a member of the immunoglobulin superfamily including at least two activating receptors, namely TREM-1 and TREM-2,\textsuperscript{17} as well as an inhibitory receptor called TREM-like transcript (TILT)-1.\textsuperscript{17} TREM-1 is a pattern-recognition receptor expressed on polymorphonuclear granulocytes and mature monocytes;\textsuperscript{68} sTREM-1 is a soluble form of TREM-1 that may be released into body fluids.
upon the upregulated expression of TREM-1 during bacterial or fungal infections. Various diseases and clinical conditions such as sepsis, pneumonia, pleural effusion, septic arthritis, meningitis, peritonitis, uterine cavity infection, etc., induce significant increase in suTREM-1 body fluids levels, suggesting that suTREM-1 could be considered a valuable biomarker for making distinctions between infectious and non-infectious diseases. Recently, urine suTREM-1 has been found to play a role in the early diagnosis of sepsis and sepsis-induced AKI. Changes in urine suTREM-1 may be an aid to distinguish severity of sepsis, and be more accurate and sensitive than traditional indicators for the dynamic assessments of prognosis. Increased suTREM-1 serum levels have been also found in infectious shock patients, being closely correlated with the severity of infection. Mostly, suTREM-1 serum dynamic changes accurately predict patient outcome and can be effectively used as prognostic indicators. A 4.5 h solid-phase double antibody ELISA designed to measure human TREM-1 in cell culture supernates, serum, plasma, and saliva is commercially available (R&D Systems, Inc., Minneapolis, MN, USA).

Inflammation and immune response against infection, as well as cancer invasiveness and tissue remodeling following injury, are essentially characterized by a highly coordinated multistep process leading to cell migration across the blood–barrier and into tissues. Thus, cell migration is tightly linked to adhesion and chemotaxis, which are mediated by a functional interaction between integrins and the urokinase-type plasminogen activator receptor (uPAR). uPAR is a glycosyl-phosphatidylinositol (GPI)-linked membrane protein consisting of three globule-like domains and can be cleaved and released from the cell membrane by GPI-specific phospholipase C or D to form soluble uPAR (suPAR). suPAR is commonly detectable in various body fluids, including plasma, urine and cerebrospinal fluid (CSF). suPAR expression is increased in cytokine or bacteria-activated macrophages, monocytes, and various immunologically active cell populations (endothelial cells, fibroblasts, smooth muscle cells, etc.), contributing to the infiltration of inflammatory cells into infected tissues or organs. On the basis of results reported by several clinical studies, it appears that suPAR is of limited value as a diagnostic biomarker of sepsis, being less accurate than CRP and PCT. However, high suPAR plasma levels closely correlate with morbidity and mortality in septic patients, demonstrating its value as prognostic biomarkers in systemic inflammation and sepsis. Finally, data from the literature suggest that during the follow-up of septic patients, changes in suPAR serum concentration may adequately reflect the response to therapeutic treatment, even if these results should be validated by further larger, multicenter trials. Analytical methods for measuring suPAR are commercially available, as reported elsewhere; in healthy adults, reference values range from 0.92 to 1.8 µg/L, with a median value of 1.2 µg/L; suPAR serum levels seem not to be influenced by age. A commercial ELISA has been developed and validated for the selective measurement of the complex suPAR:PAI-1 (specific high-affinity plasminogen activator inhibitor-1). 7. Managing necrotizing enterocolitis: challenges in biomarker research

Necrotizing enterocolitis (NEC) is a disease of the infant gastrointestinal tract, most commonly found in VLBW premature babies and often occurring in the first four postnatal weeks. IUGR may be an additional specific risk factor, especially if associated with circulatory redistribution demonstrated by absent or reversed end-diastolic flow velocities in antenatal Doppler studies of the fetal aorta or umbilical artery. The rapid evolution of NEC from bacterial invasion of the intestinal wall to full-thickness bowel necrosis leads to perforation and subsequent peritonitis, sepsis, and ultimately to death. In newborns with a birth weight ranging 500–1500 g, the mean prevalence of the disorder is about 7.0%. NEC is one of the leading causes of morbidity and mortality in NICUs globally: the estimated rate of death associated with necrotizing enterocolitis ranges between 20% and 30%, with the highest rate among infants requiring surgery. This is mainly due to the elusive nature, the unpredictable onset and progression, and the fragile nature of the affected patient population. In NEC, the excessive inflammatory process initiated in the highly immunoreactive intestine extends the effects of the disease systemically, affecting distant organs such as the brain and placing affected infants at substantially increased risk for neurodevelopmental delays. Despite the importance of an abnormal microbial colonization in the intestine and a highly immunoreactive intestinal mucosa as main factors involved in the pathogenesis of NEC, several predisposing factors significantly participate in the pathophysiology of classic NEC, such as genetic predisposition, intestinal immaturity, and an imbalance in microvascular tone.

The evaluation and screening of NEC is widely based on conventional biomarkers such as CRP and platelet-activating factor (PAF). Unfortunately, CRP is a sensitive but highly non-specific marker of NEC, resulting in poor provision of specific diagnosis and guide therapy, especially in the early stages of the disease. Encouraging results have been reported in the literature on PAF sensitivity and specificity in diagnosing NEC; however, a number of limitations affecting published studies (definition of controls, time of samples collection in the course of the disease, etc.) together with the absence of large validation trials reduce the potential clinical power of PAF as useful biomarker for NEC diagnosis and follow-up.

Emerging candidate biomarkers for NEC include Claudin-3, the proteins intestinal- and liver-specific fatty acid-binding proteins (IFABP), and fecal calprotectin. In particular, fecal calprotectin seems to be of great clinical interest, having been previously validated as an accurate marker of inflammatory bowel disease in both adults (Crohn’s disease, ulcerative colitis, etc.) and children (chronic diarrhea, etc.). Calprotectin was originally discovered as an antimicrobial protein that was present in the cytoplasm of neutrophil granulocytes; it is a calcium- and zinc-binding protein constituting ~60 per cent of the cytosolic protein of neutrophils, monocytes, and macrophages. Calprotectin is released upon inflammatory activation in the gut and thus is detectable in both the feces and plasma. Fecal calprotectin has been found significantly increased in VLBW babies diagnosed with NEC; recently, it was reported that in VLBW babies with gastroinestinal injury or infection, fecal calprotectin concentration is higher than in those with other minor systemic infection or stress. Moreover, it was found that fecal calprotectin decreases as these illnesses resolved. Despite the small number of cases reported in the literature, fecal calprotectin shows some promise as biomarker for NEC, supported by a favorable disease likelihood ratio, a positive correlation with the severity of the disease, and a close association between its decrease and the positive response to therapy. Limitations in diagnostic efficiency of fecal calprotectin originate from variables affecting its levels, such as gestational and postnatal age; they may be the cause of unusually low fecal calprotectin levels observed in such cases of fulminant NEC.

8. Metabolomics: a tool for discovering future biomarkers

Metabolomics, also called ‘the new biochemistry’, is a holistic approach based on the systematic study of the complete set of metabolites (metabolome) contained in a biological sample. The ‘essence’ of metabolomics is the important concept that the
metabolic status of the individual is the accurate representation of the state of health or illness of the individual itself. Therefore, the metabolome is considered the phenotype reflecting even the epigenetic modifications. The \[^1H\]NMR (nuclear magnetic resonance) metabolic analysis of various biological fluids or tissues has been used successfully in the fields of physiology, diagnostics, pharmacology, toxicology and nutrition, facilitating the discovery of molecules associated with these processes.

However, the introduction of new biomarkers for neonatal diseases in clinical practice remains a problematic task, because of the continuous rapid, abrupt changes in neonatal physiology over the first month of life. One of the most variable physiological parameters is the body water composition: there are significant differences between premature infants (85% body water), infants (75% body water) and adults (50–60%). In addition, in newborns the extracellular water is 40% of total body weight compared with 20% in adults. Glomerular filtration rate is also lower in preterm infants than in full-term infants as well as than in adults, and this restriction persists throughout the first weeks of life.

There are several biological fluids that can be used to study the metabolome, such as urine, blood, saliva, cerebrospinal fluid. However, urine can be considered the best sample at least for two simple reasons: (i) urine is easy to collect and permits an non-invasive approach for measuring biological substances; (ii) urine can be considered a complex biological fluid, containing a multitude of biological metabolites and, more specifically, the intermediate metabolites, which reflect specific metabolic processes related to the current state of health or disease in real time. Urine can also be considered an open window on what happens in the body, representing an ideal ‘open system’ by which we can observe various physiological processes such as balancing water and salt composition, metabolic degradation, elimination of harmful or toxic substances, and maintaining homeostasis. In addition, once urine samples are collected they can be stored in a freezer at −80 °C until the analysis.

All this makes the urine a biological fluid particularly appropriate to study and to monitor what happens at the molecular and metabolic level in the body. The technique consists of two sequential steps: (i) an experimental technique, based on the nuclear magnetic resonance analysis (NMR) and/or mass spectrometry analysis (MS) in order to create a profile of the compounds contained in the samples, and (ii) a multivariate analysis of the data obtained. The results are usually shown graphically to screen the samples in space that highlight the components that contribute to the highest intrinsic variations of the groups considered. The metabolites corresponding to the discriminant variations will be identified.

The application of metabolomics in biomarker discovery in the perinatal period has been performed recently in a group of newborns with IUGR, which is defined as failure of a fetus to achieve its full potential growth at birth. It is a complex disorder characterized by a weight and body mass lower than normal, hypoglycemia, kidney deficiency, respiratory distress, NEC, and intracranial hemorrhage. However, information regarding the metabolic profile of IUGR is fragmented in spite of it being one of the most common problems in the care of premature infants. A study was performed in two groups of infants. The first group consisted of preterm newborns with IUGR diagnosed by ultrasonography during the fetal period. The second group consisted of preterm neonates of suitable weight for their gestational age at birth as a control. A urine sample was collected from each newborn at birth, and was analyzed using \[^1H\]NMR. The analysis of the two populations was statistically significantly different, revealing an increased flux of the urea cycle, amino acid metabolism, glycine, serine and threonine metabolism in the IUGR group. This metabolic profile of IUGR appears to be associated with a significant increase of myoinositol levels (P = 0.04) compared with the control group.

In accordance with this result, several published studies using animal models of IUGR, newborn population or cell culture highlight an increased level of myoinositol in the placenta, fetal arterial plasma, plasma and fibroblast. Though the role of myoinositol is still unclear, it seems to be associated with the development of metabolic syndrome. In conclusion, metabolomics appears to be a predictive tool which may be used to characterize the IUGR group.

In conclusion, the increasing speed of knowledge in basic biological research together with the rapid development of high-technology diagnostic systems (e.g. nanotechnologies, microarrays, etc.) significantly contributes to gains in neonatal life expectancy. However, new efforts are needed to reduce the growing gap between research and clinical practice. Progress in translational research could contribute to develop and commercialize new low-cost medical devices that are also easily usable in low-income countries; for example, metabolomics may have the extraordinary ability to identify a panel of metabolites clinically significant in acute or chronic neonatal clinical conditions. Once adequately validated, health care industries can develop a low-cost device for the identification of these metabolites by a simple, inexpensive reaction between a drop of blood or urine and specific lyophilized reagents on a treated chemical strip, similarly to dipsticks often used for urinalysis. Therefore, targeted investments in research and high-technology diagnostic systems may represent a valid opportunity for improving neonatal care, appropriateness in therapeutic treatment, and outcome and for reducing health care expenditure worldwide.

**Practice points**

- In 2010, neonatal mortality was estimated to be 23 deaths per 1000 live births worldwide equal to 3.06 million babies dying in the neonatal period.
- 75% of all neonatal deaths occur within the first seven days of life and almost 33% of these babies die during the first 24 h of life.
- Infections, asphyxia, and preterm birth, together account for nearly 80% of these deaths.
- Every year, almost one million newborns die from infections, mostly in low-income countries.
- A great deal of effort in reducing neonatal mortality rate is put into searching for new biochemical markers.
- C-reactive protein (CRP) is a very specific, but not very sensitive, biomarker of bacterial infection and the evolution of sepsis is poorly correlated with changes in serum CRP level.
- Serum procalcitonin (PCT) increases within 4 h from the start of the innate immunity cascade, peaking within 6–8 h, but has dynamic reference intervals and cut-off ranges.
- Fundamental research driven by scientists’ interest in discovering pathways involved in the innate immunity and in endothelial barrier disruption have resulted in the evaluation of a new generation of biomarkers for sepsis.
- The most interesting and reliable candidate biomarkers for managing neonatal sepsis may be: soluble CD14 subtype presepin (sCD14-ST), lipopolysaccharide binding protein (LBP), angiopeptins (Ang) -1 and -2, the triggering receptor expressed on myeloid cells (TREM-1), the soluble urokinase-type plasminogen activator receptor (suPAR), platelet-activating factor (PAF) and calprotectin.
- Metabolomics represents the passage from a descriptive science to a predictive science, having the potential to translate benchtop research into real clinical benefits.
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