Review

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Early predictors of perinatal brain damage: the role of neurobiomarkers

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Abstract: The early detection of perinatal brain damage in preterm and term newborns (i.e. intraventricular hemorrhage, periventricular leukomalacia and perinatal asphyxia) still constitute an unsolved issue. To date, despite technological improvement in standard perinatal monitoring procedures, decreasing the incidence of perinatal mortality, the perinatal morbidity pattern has a flat trend. Against this background, the measurement of brain constituents could be particularly useful in the early detection of cases at risk for short-/long-term brain injury. On this scenario, the main European and US international health-care institutions promoted perinatal clinical and experimental neuroprotection research projects aimed at validating and including a panel of biomarkers in the clinical guidelines. Although this is a promising attempt, there are several limitations that do not allow biomarkers to be included in standard monitoring procedures. The main limitations are: (i) the heterogeneity of neurological complications in the perinatal period, (ii) the small cohort sizes, (iii) the lack of multicenter investigations, (iv) the different techniques for neurobiomarkers assessment, (iv) the lack of consensus for the validation of assays in biological fluids such as urine and saliva, and (v), the lack of reference curves according to measurement technique and biological fluid. In the present review we offer an up-to-date overview of the most promising developments in the use of biomarkers in the perinatal period such as calcium binding proteins (S100B protein), vasoactive agents (adrenomedullin), brain biomarkers (activin A, neuron specific enolase, glial fibrillary acidic protein, ubiquitin carboxyl-terminal hydrolase-L1) and oxidative stress markers.

Keywords: biomarker; brain damage; hypothermia; newborn; perinatal asphyxia; S100B.

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Introduction

There has been a dramatic increase in recent years in the number of studies focusing on neurobiomarkers (NBs) of central nervous system (CNS) injury [1–11]. The optimal NB should be reliable, easily and harmless to collect, reproducible and able to guide caregivers in daily practice. The Food and Drug Administration (FDA), the European Medicines Agency (EMA) and the National Institutes of Health (NIH) are supportive of research into biomarkers in order to: (i) improve the early identification of cases at risk, (ii) promote specific preventive or therapeutic treatments [2], and (iii) provide health care professionals with a new tool, as well as to set the stage for a more modernized standard of care for the testing of suspect cases (Table 1).

The optimal NB should monitor disease progression by longitudinal assessments and possibly correlate with standard procedures such as cerebral ultrasound (CUS) and magnetic resonance imaging (MRI) to assess the entity of brain injury. The ranges of normality of the ideal NB should be available for both healthy term and preterm neonates, and possibly identifiable in different biologic fluids (amniotic; cerebrospinal fluid [CSF]; blood; urine;

Table 1: Criteria for an NB inclusion in clinical guidelines according to the FDA and EMA statements.

Optimality items for an NB according to the FDA and EMA
1. Alternative and direct indicator of CNS damage when clinical and radiologic assessments are still silent
2. Early predictor of degree and location of injury
3. Indicator of the extent of brain lesion
4. Monitor the progression of disease
5. Well studied in the pediatric population
6. Measurable by available commercially kits worldwide with good reproducibility
7. Presence of reference range for the pediatric population
8. Assessment in different biological fluids (urine, blood, CSF, amniotic fluid, saliva, milk)

CSF, cerebrospinal fluid; CNS, central nervous system; EMA, European Medicines Agency; FDA, Food and Drug Administration; NB, neurobiomarker.

saliva; milk). However, to date only a few NBs meet these criteria for routine use [2].

The present review offers an up-to-date overview of the most promising developments regarding the use of NBs closer to FDA and EMA criteria, such as S100B protein, adrenomedullin (AM), activin A (AcA), neuron specific enolase (NSE), oxidative stress markers (OSM), glial fibrillary acidic protein (GFAP) and ubiquitin carboxyl-terminal hydrolase-L1 (UCH-L1), in the perinatal period. This field is particularly challenging due to the heterogeneity of neurological complications which may complicate the early life periods.

S100B

S100B protein and its heterodimeric form ββ is highly specific for the CNS being expressed mostly in glial cells but also detectable in neuronal subpopulations [12–14]. S100B has a half-life of 1-h and is eliminated mostly by the kidneys (98%) [15]. S100B has been detected in different biological fluids such as amniotic, CSF, blood, urine, saliva and milk [16–23]. It has neurotrophic effects at physiologic levels (nanomolar), but becomes neurotoxic at high concentrations (micromolar) [24–27].

CSF

The first studies investigating S100B as a perinatal NB were performed using CSF. Whitelaw et al. showed that S100B was higher than controls in preterm newborns with post-hemorrhagic ventricular dilatation [28, 29].

In term neonates, Blennow et al. found that S100B in the CSF of term newborns, complicated by perinatal asphyxia (PA), correlated significantly with neurological impairment at 1-year of age, or death before that time [30]. These results were therefore comparable to those recorded in preterm newborns [28, 29]. However, because of ethical and medical issues and the impossibility of longitudinal monitoring, the assessment of S100B in CSF was progressively abandoned.

Blood

Following previous observations in CSF, S100B was measured in blood. Among a panel of biomarkers, S100B had the highest sensitivity in cord blood for the prediction of brain injury in preterm infants. S100B at a cut-off of 1.07 μg/L achieved a specificity of 53% and a sensitivity of 93% as a predictor of brain damage in preterm infants [23] (Table 2).

Conversely, Costantine et al. showed that the predictive value of S100B for the development of cerebral palsy was weaker after adjusting for gestational age (GA) and perinatal treatment such as magnesium sulfate [57].

Increased S100B peripheral blood levels were also observed in: (i) 24 preterm newborns complicated by intraventricular hemorrhage (IVH) 48 h before the development of any clinical, laboratory or ultrasonographic sign of hemorrhage. A correlation between S100B and the extent of IVH was also reported [58]; (ii) in preterm newborns complicated by intrauterine growth restriction (IUGR), presumably as a consequence of the hemodynamic re-arrangement [59–61].

In term newborns, increased concentrations of serum S100B have been observed in PA infants complicated by hypoxic-ischemic encephalopathy (HIE). Nagdyman et al. measured cord blood concentrations of a panel of NBs including S100B, in a cohort of 49 term newborns. They found higher S100B in PA infants with moderate-severe HIE at 2 h after birth. Moreover, a combination of S100B

Table 2: Currently available studies providing data about NBs availability as predictors of brain damage and/or ominous outcome.

Biomarker	Fluid	Disease	Cut-off	PPV, %	NPV, %	Specificity, %	Sensitivity, %	Reference
S100B	CB	PA-HIE	8.5 μg/L	71	90	90	71	[31]
	CB	PN, BD	1.07 μg/L	NA	NA	52.9	95.3	[23]
	PB	PA-HIE	1.6 ng/mL	NA	NA	91	40	[32]
	U	PN-IVH	0.70 μg/L	NA	NA	100	100	[33]
	U	IUGR-BD	7.37 MoM	100.7[a]	0.05[a]	99.1	95	[34]
	U	PN-D	12.93 MoM	78.6	100	97.8	100	[35]
	U	PA-BD	0.28 μg/L	46.2	100	87.3	100	[36]
	U	PA-HIE	0.41 μg/L	80.8	97.8	94.6	91.3	[37]
	U	TN-D	1.0 μg/L	100	100	100	100	[38]
	U	TN-D	1.11 μg/L	NA	NA	60	100	[39]
	U	TN-BD	0.66 μg/L	NA	NA	70	83	[39]
	S	CHD-BD	3.25 MoM	100	100	100	100	[40]
AM	PB	CHD-BD	17.4 ng/L	3.7[a]	0.0[a]	73	100	[41]
	AB	CHD-LCOS	27.0 pg/L	39.1	100	64.1	100	[42]
AcA	CSF	PA-HIE	1.3 ng/L	100	0	100	100	[43]
	CB	PN-IVH	0.8 μg/L	79	100	93	100	[44]
	CB	PN-BD	321 ng/L	NA	NA	92	86	[23]
	PB	PA-HIE	0.66 ng/L	27.69[a]	0.069[a]	96	93	[45]
	PB	CHD-BD	0.94 ng/L	100	0	100	100	[46]
	U	PN-IVH	0.08 ng/L	45.8	93.4	84.5	68.7	[47]
	U	PA-HIE	0.08 ng/L	NA	NA	100	83	[48]
NSE	CB	PA-HIE	44.0 μg/L	46	93	83	68	[31]
	PB	PA-HIE	40.0 μg/L	51	89	70	79	[49]
	PB	PA-BD	45.4 μg/L	39	95	70	84	[49]
	PB	PA-HIE	81.0 ng/mL	NA	NA	83	71	[32]
OS	CB	TN-PN-BD	15.2 μmol/L	NA	NA	100	100	[50]
G-FAP	CB	PN-PVL-WM	>0.04 ng/mL	NA	NA	91.2	52.4	[51]
	PB	PA-HIE	>0.08 ng/mL	NA	NA	100	100	[52]
	PB	PA, BD, D	0.2 ng/mL	78	90	82	87.5	[53]
	PB	PA, HIE	0.07 ng/mL	NA	NA	78	77	[54]
UCH-L1	PB	PA, BD, D	13.8 ng/mL	78	90	100	75	[53]
	PB	PA, HIE	18.0 ng/mL	NA	NA	NA	NA	[55]
	PB	PA, HIE, BD	28.0 ng/mL	NA	NA	95	NA	[56]

AcA, activin A; AM, adrenomedullin; BD, brain damage; CSF, cerebrospinal fluid; BF, biologial fluid; CB, cord blood; D, early neonatal death; G-FAP, glial fibrillary acid protein; HIE, hypoxic ischemic encephalopathy; IVH, intraventricular haemorrhage; MB, maternal blood; NPV, negative predictive value; NSE, neuron specific enolase; OSM, oxidative stress markers; PB, peripheral blood; PN, preterm neonate; PPV, positive predictive value; PVL, periventricular malacia; S, saliva; TN, term neonate; UCH-L1, ubiquitin carboxyl-terminal hydrolase L1; U, urine; WM, white matter injury. [a]Likelihood ratio (positive/negative).

(cut-off value: 8.5 μg/L) and CK-BB (cut-off value: 18.8 UI/L) as early as 2 h after birth had the highest sensitivity (83%) and specificity (95%) in predicting moderate and severe HIE [31].

S100B cord blood levels were also assessed in 13 term infants with stage II–III HIE and compared to 21 healthy controls. Higher S100B concentrations were associated with cord blood acidosis, aEEG pattern severity, HIE severity, and correlated with neurodevelopmental sequelae at 6-year follow-up and death) [62]. In contrast, Summanen et al. found cord blood S100B to be a poor NB of PA in term newborns [63] (Table 2).

Lastly, in a cohort of 100 term newborns of whom 20 complicated by PA-HIE and intracranial hemorrhage (ICH), S100B was significantly higher in the PA-ICH group than in those who did not develop ICH and in controls [64].

Urine

In preterm infants, urine S100B levels were longitudinally measured in the first 72 h from birth in a cohort of 36 cases, of whom 18 developed IVH. Increased S100B at birth was detected 72 h before the development of any clinical, laboratory or ultrasonographic sign of IVH. At a cut-off of 0.70 μg/L, the sensitivity and specificity of urinary S100B as a diagnostic test were 100%. S100B also correlated with

the extent of IVH by means of longitudinal CUS recordings [33] (Table 2).

In IUGR infants, S100B was measured longitudinally in the first 7 days from birth in a cohort of 84 infants, of whom 42 were growth restricted. S100B was higher at all monitoring time-points in the IUGR newborns showing an abnormal neurological outcome than in those who did not and in controls. At a cut-off of 7.37 multiples of median (MoM) at first urination, S100B achieved a sensitivity of 95% and a specificity of 99.1% as a single marker for predicting an adverse short-term neurological outcome [34] (Table 2).

S100B was also longitudinally measured (in the first 96 h from birth) in 165 preterm newborns of whom 11 suffered early neonatal death, 121 displayed no overt neurologic syndrome, and 33 suffered neonatal hypoxia and IVH but no ominous outcome. S100B concentration was higher at all monitoring time-points in preterm newborns who later died than in the other studied groups. At a cut-off of 12.93 MoM at first void, sensitivity was 100%, specificity 97.8%, and positive (PPV) and negative predictive values (NPV) 78.6% and 100%, respectively, for predicting early post-natal death [35] (Table 2).

In a cohort of 277 late preterm infants, S100B was found to be gender- and GA-dependent [65].

In PA infants, urine S100B was longitudinally measured in the first 72 h from birth in a cohort of 134 term newborns, of whom 38 were complicated by PA-HIE with normal (n = 20) or abnormal (n = 18) 1 year neurological follow-up and 96 controls. S100B was higher in PA infants developing an abnormal neurological outcome than in normal PA infants and controls. At a cut-off value of 0.28 μg/L at first urination, S100B achieved a sensitivity of 100% and a specificity of 87.3% for predicting an adverse neurological follow-up [36]. The same authors also found higher S100B in severe PA-HIE infants than in mild PA-HIE infants and controls. An S100B concentration cut-off of 0.41 μg/L at first urination had a sensitivity of 91.3%, a specificity of 94.6%, a PPV of 80.8% and an NPV of 97.8% as a predictor of HIE [37] (Table 2).

As for preterm newborns, the diagnostic accuracy of S100B in predicting early postnatal death was investigated in a cohort of 132 term newborns, of whom 48 had PA-HIE and 12 died within 7 days from birth. Higher S100B was observed in the PA-HIE newborns who died than in the other groups studied. At a cut-off of 1.0 μg/L S100B had a sensitivity/specificity, PPV and NPV of 100% for predicting neonatal death [38] (Table 2).

Finally, Alshweki et al. measured S100B in the urine of 31 PA-HIE newborns with normal (n = 13) and abnormal/ominous outcome (n = 18) detected by MRI. Higher S100B was observed in infants with unfavorable outcome. An S100B cut-off of 1.11 μg/L had a sensitivity of 100% and a specificity of 60% for the prediction of neonatal death, whilst at a cut-off of 0.66 μg/L S100B had a sensitivity of 83% and a specificity of 70% for the prediction of abnormal neurological outcome [39] (Table 2).

Saliva

Data in the literature show that S100B: (i) is essentially absent from fetal salivary glands [66], (ii) is not produced by salivary glands, and that (iii) saliva concentrations are derived from systemic circulation [67]. A reference curve of S100B in saliva was therefore provided in a cohort of 216 preterm and term newborns. The results showed, as for other biological fluids (cord blood, urine), that S100B was higher in preterm than in term newborns and was GA-dependent [21]. The findings allowed the identification of saliva as offering the least stressful fluid for CNS clinical monitoring in a neonatal intensive care unit (NICU).

In an international multicenter study, recruiting a cohort of 292 term newborns, of whom 48 suffered PA and 244 healthy controls longitudinal S100B saliva concentrations correlated with the presence of neurological abnormalities at 1 year after birth. S100B was higher in PA newborns with poor prognosis than in PA with good prognosis or controls. At a cut-off >3.25 MoM, S100B achieved a sensitivity/specificity of 100% for predicting the occurrence of abnormal MRI patterns and neurological outcome [40] (Table 2).

S100B and perinatal therapeutic strategies

S100B has also been investigated to evaluate the *pros* and *cons* of *in utero* and post-natal therapeutic strategies such as antenatal maternal glucocorticoid (GC) treatment, nitric oxide donor (NO) and selective serotonin re-uptake inhibitors (SSRI) [68–70].

GC supplementation is widely used for the prevention of lung immaturity, but no conclusive data on its possible side-effects on other organs, including the CNS, are available [71]. In this regard, preterm newborns, antenatally GC-treated showed lower longitudinal urinary S100B levels (in the first 120 h from birth) than in controls [68]. The findings are supportive of an inhibited release of S100B as a neurotrophic factor, offering additional evidence in the debate on the side-effects on CNS development of GC. Notably, Sannia et al. found that S100B levels, measured in the first 72 h changed

in a GC dose-dependent manner in preterm newborns whose mothers received a complete course of GC than in infants whose mothers received half a course or no GC [72]. In recent decades, the effects of NO in IUGR fetuses with placental insufficiency have been investigated in a randomized controlled multicenter trial admitting to the study 48 IUGR pregnancies treated with either placebo (n = 25) or a transdermal glyceryl-trinitrate patch (5 mg every 16 h daily) until delivery (n = 23). S100B cord blood levels were lower in NO-treated pregnancies than in the placebo group and an improved neurological and respiratory outcome was also observed [69].

Finally, the use of SSRI during pregnancy is increasing both in Europe (2%–3%) and in the USA (8%–10%) [73]. This trend has evolved without any solid evidence on the safety or efficacy of this approach, and treatment is recommended for pregnant women with depression despite the potential side-effects on the fetus and newborn [73–76]. In light of this, S100B was assessed in 306 pregnant women, of whom 75 were SSRI-treated, and 231 healthy controls, and their newborns. The results showed higher S100B in maternal-fetal-neonatal fluids in the SSRI-treated group, particularly in infants with 7-day adverse neurological outcome [70].

S100B and hypothermia

Today, hypothermia (HT) is an accepted standard of care therapeutic strategy for term newborns complicated by PA-HIE [77, 78]. Longitudinal blood levels of a panel of NBs, including S100B, were measured during HT in a cohort of 83 PA-HIE infants. S100B increased during HT and was associated with adverse neurodevelopmental outcome at 15 months of age [79]. Similarly, Massaro et al. correlated S100B with cerebral MRI patterns at 14 day neurological follow-up in 75 HT-treated PA-HIE infants. They found higher S100B in term newborns with unfavorable outcome and pathological MRI patterns. S100B at a cut-off of 1.6 ng/mL reached a specificity and a sensitivity of 91% and 40%, respectively, as a diagnostic test of brain injury [32] (Table 2). These data partly agreed with those of Roka et al. [80], who found, in a cohort of 24 HT (n = 13) or non-HT-treated (n = 11) PA-HIE infants, elevated S100B in both HT and no-HT groups.

Recently, the potential neuroprotective effects of erythropoietin were evaluated in 50 HT-treated PA-HIE infants, using a panel of NBs including S100B. The protein increased in infants showing pathological cerebral MRI patterns. No significant effects of erythropoietin treatment on NB levels were detectable [81].

HT can be performed as selective head cooling or whole-body cooling. Celik et al. compared the efficacy of the two HT procedures using a panel of NBs including S100B in 21 PA-HIE infants. Neurological follow-up was set at 1 year after birth. The results showed no differences in NBs between the two HT methods [82].

AM

AM, first isolated from human pheochromocytome, is a vasodilator peptide detectable in multiple tissues [83–85]. AM's half-life is 2 h and it is eliminated mostly by the kidneys [86]. Both *in vitro* and *in vivo*, AM: (i) influences local and systemic blood pressure [87], (ii) plays a special role in the regulation of blood flow [88], (iii) has a role in cardiovascular adaptation after birth [89–91], (iv) is released after stressful events such as hypoxia and PA [91, 92], and (v) as a neuropeptide, regulates cerebral blood flow [93, 94].

CSF

To the best of our knowledge, no studies have measured AM in the CSF of preterm newborns. In term newborns and children, higher levels of AM were found in the CSF after traumatic brain injury as an endogenous response to cerebral hypoperfusion [93].

Blood

In cord blood, AM was measured in IUGR (n = 16) and non-IUGR (n = 16) infants. Higher AM was found in IUGR infants than in controls, suggesting that AM acts as a compensatory mechanism in response to chronic hypoxia and its hemodynamic re-arrangement characterized by the brain-sparing effect [95].

In venous blood of preterm newborns developing IVH (n = 24) and controls (n = 48), higher levels of AM were found within 6 h from birth, suggesting the involvement of AM in the loss of cerebral vascular regulation secondary to a hypoxic insult [96].

In term newborns, Kamata et al. found that cord AM levels in the umbilical artery and vein were higher in infants with persistent pulmonary hypertension (n = 15) than in controls (n = 8) [97]. In PA newborns developing IVH (n = 20) and in controls (n = 20), higher AM was observed in PA-IVH infants, suggesting its involvement in the loss of cerebral vascular regulation due to HI [98].

AM has also been measured in a cohort of 80 infants complicated by significant hyperbilirubinemia (n = 40) or non-significant hyperbilirubinemia (n = 40). Higher AM was observed in infants with significant jaundice, suggesting its involvement in adverse effects and neuronal injury steps of hyperbilirubinemia [99].

In congenital heart disease (CHD) term newborns complicated (n = 40) or not (n = 10) by adverse 1-year neurological outcome, decreased AM levels were observed in neurologically abnormal infants. At a cut-off of 17.4 ng/L, AM as a predictor of neurological abnormalities achieved a sensitivity of 100%, a specificity of 73.0%, and positive (PLHr) and negative (NLHr) likelihood ratios of 3.7 and 0.0, respectively [41]. Furthermore, in CHD infants complicated (n = 9) or not (n = 48) by intra-operative low cardiac output syndrome (LCOS), Abella et al. found lower AM in LCOS infants. At a 27 pg/L cut-off, AM achieved a sensitivity of 100%, a specificity of 64.1%, a PPV of 39.1% and a NPV of 100.0% for the prediction of LCOS [42] (Table 2).

AcA

AcA is a member of the TGF-beta superfamily involved in the regulation of a variety of functions, including cell proliferation, differentiation, bone remodeling, hematopoiesis, wound healing and apoptosis [100, 101]. AcA promotes neuronal differentiation, and increased AcA levels in biological fluids have been found under HI conditions, as a response to brain injury, and as a local mediator of angiogenesis during the repair process [102, 103].

AcA also has a beneficial role in neuronal recovery, supporting the survival of neurogenic cell lines and retinal neurons [104], and offering protection against neurotoxins of different origins [105]. In animal models, AcA enhanced the survival of embryonic hippocampal neurons [106], to decrease ischemic brain injury and rescue striatal neurons against neurotoxic damage [107].

CSF

To the best of our knowledge, no studies have measured AcA in the CSF of preterm newborns.

AcA was assessed within 24 h in the CSF of 74 term newborns, of whom 30 were PA newborns and 44 controls. Higher AcA was found in infants affected by severe HIE than in moderate HIE and controls. At the cut-off of 1.3 ng/L, AcA reached a sensitivity and specificity of 100%, a PPV of 100% and an NPV of 0% as a predictor of HIE [43] (Table 2).

Blood

Florio et al. measured AcA in cord blood of hypoxic (n = 26) and non-hypoxic (n = 24) preterm newborns. Higher AcA was found in hypoxic preterm newborns than in controls. The main explanations is that AcA can act as a biomarker of hypoxia and/or be an expression of neuroprotective process activation [108]. The same authors measured cord blood AcA levels in 50 high-risk pregnancies and 40 controls. Higher AcA was observed in high-risk newborns and correlated with the occurrence of the brain-sparing effect and the length of hospital stays [109].

In preterm newborns developing (n = 11) or not (n = 42) IVH, AcA was measured in arterial cord blood soon after birth (2-h). Higher AcA was observed in IVH preterm newborns than in controls. At a cut-off of 0.8 μg/L, AcA has a sensitivity of 100%, a specificity of 93%, a PPV of 79% and an NPV of 100% as a predictor of IVH [44] (Table 2).

In a cohort of 130 preterm infants, Lu et al. measured amniotic and cord blood levels of a panel of biomarkers (AcA, interleukin-1β, interleukin-6, interleukin-8, tumor necrosis factor-α, granulocyte colony-stimulating factor, monocyte chemotactic protein-1, soluble intercellular adhesion molecule-1, S100B) among which AcA was the best predictor of long-term brain injury. AcA at a cut-off of 321 ng/L achieved a specificity of 92% and a sensitivity of 86% as a predictor of brain damage in preterm infants [23] (Table 2).

AcA concentrations in cord blood were assessed in 26 hypoxic infants and 60 controls. AcA was higher in hypoxic preterm newborns in a gender-dependent manner [110].

In blood, AcA was longitudinally assessed in 35 PA-HIE newborns and 70 controls. AcA was higher in infants affected by severe HIE than in moderate-HIE infants and controls. At the cut-off of 0.66 ng/L, AcA achieved, as a predictor of HIE, a sensitivity of 93%, a specificity of 96%, a PLHr of 27.69 and NLHr of 0.069 [45] (Table 2).

AcA was measured in the perioperative period in 45 CHD infants, of whom 36 were without overt neurologic injury and nine had a neurologic injury at 7 days after surgery. Higher AcA in the perioperative period was found in neurologically abnormal than in neurologically normal infants. At a cut-off of 0.94 ng/L, AcA had a sensitivity and specificity of 100% for predicting perioperative neurological abnormalities [46] (Table 2).

Urine

In preterm newborns developing (n = 20) or not (n = 80) IVH, AcA was longitudinally measured in urine soon after birth (2-h). Higher AcA was observed in IVH preterm newborns than in controls. At a cut-off of 0.08 ng/L, at the first void, AcA had a sensitivity of 68.7%, a specificity of 84.5%, PPV and NPV 46%–93%, respectively, as a predictor of IVH [47] (Table 2).

In urine, AcA was longitudinally assessed in 30 PA-HIE infants and 30 controls. Higher AcA was found in infants affected by severe-HIE than in moderate-HIE and controls. At a cut-off of 0.08 μg/L, AcA achieved, as a predictor of HIE, a sensitivity of 83% and a specificity of 100% [48] (Table 2).

AcA and perinatal therapeutic strategies

AcA concentrations in maternal and fetal biological fluids were assessed in 24 mothers antenatally treated with SSRI and in 24 controls. Higher AcA was observed in maternal and fetal biological fluids in SSRI-treated subjects than in controls. The authors suggested that AcA may play a key role as a new therapeutic option and/or marker of maternal/fetal CNS stress in pregnant women with depression [111].

NSE

NSE, a glycolytic enzyme detected at high concentrations in neuronal cytoplasm [112], represents a late marker of neural differentiation and maturation. NSE is released into the extracellular space in cases of cell death [113].

CSF

In children, NSE increases in both CSF and blood after impairment of the blood-brain barrier and brain injury [114–116]. Few studies have investigated the role of NSE in neonates.

Increased NSE in CSF was observed in PA-HIE term newborns compared with controls [117–119].

Blood

Costantine et al. performed a multicenter randomized trial of magnesium sulfate administration vs. placebo administration to prevent cerebral palsy development or death in preterm infants. The results showed that NSE cord blood levels were unable to differentiate preterm infants who developed cerebral palsy (n = 16) or died within 1 year of age (n = 25) from those who did not [57].

In the blood of 18 CHD infants undergoing open-heart surgery and cardiopulmonary bypass (CPB), higher NSE was observed in the perioperative period in infants with adverse neurological outcome [120].

In 30 PA-HIE term newborns, cord blood NSE levels were higher than in the 30 controls [121]. In this regard, Nagdyman et al. found higher NSE in PA infants with moderate-severe HIE (n = 7) at 2 h after birth than mild HIE (n = 22) and controls (n = 20). An NSE cut-off at 2 h of 44.0 μg/L achieved a sensitivity of 68%, a specificity of 83%, a PPV and NPV of 46%–93% in predicting moderate-severe HIE [31] (Table 2).

Higher blood NSE concentrations were observed in 43 PA-HIE infants than in controls and correlated with the severity of HIE. At a cut-off of 40.0 μg/L, NSE had a sensitivity of 79% and a specificity of 70% as a predictor of HIE. Indeed, at a cut-off of 45.4 μg/L, NSE has a sensitivity of 84% and a specificity of 70% as a predictor of poor outcome [49] (Table 2).

Finally, HT-treated PA-HIE infants showed higher levels of NSE in term newborns who developed MRI and clinical patterns suggesting brain injury [32, 80]. In particular, Massaro et al. found higher NSE concentrations in term newborns with unfavorable outcome and pathological MRI patterns. At a cut-off of 81 ng/mL, NSE reached a specificity and a sensitivity of 83% and 61%, respectively, as biomarker of brain injury [32] (Table 2). Similarly, Roka et al. found, in a cohort of 24 HT (n = 13) or non-HT-treated (n = 11) PA-HIE infants, higher NSE values in infants who died or developed severe neurological impairment [80].

OSM

It is known that the antioxidant systems of fetuses and newborns are immature, and therefore exposed to the damaging effects of oxidative stress [122]. Free radicals are highly reactive substances involved in self-amplified chain reactions leading to cell death or apoptosis. Once the balance between the production of antioxidant enzymes and of free radicals changes in favor of the latter, oxidative stress damage may occur [123]. Oxidative stress is thus involved in the pathogenesis of many fetal and neonatal diseases, mainly triggered by hypoxia.

CSF

OSM (8-isoprostane; malondialdehyde [MDA]; protein carbonyl [PC]; chlorotyrosine) were measured in 22 preterm newborns developing white matter injury, 30 term newborns and 17 adults. Higher OSM were observed in preterm newborns than in the other groups studied [124]. The same authors also showed, in a case report, increased levels of the mentioned OSM in a preterm newborn complicated by periventricular leukomalacia (PVL) [125].

No differences were found in CSF OSM (nitric oxide, nitrotyrosine) levels between mild PA-HIE term newborns (n = 11) and controls (n = 9) [126]. Lower levels of CSF lipid peroxides and antioxidant enzymes (superoxide dismutase [SOD]; glutathione peroxidase, GSHP, MDA) were found in 72 PA-HIE term newborns after high-dose administration of phenobarbital [127]. In animal models, lower MDA levels have been reported after the administration of melatonin [128].

Blood

Buonocore et al. measured plasma non-protein-bound iron (NPBI) levels in the cord blood of a cohort of 384 newborn infants of whom 51 showed an abnormal neurodevelopmental outcome. At a cut-off of 15.2 mmol/L, NPBI reached a sensitivity and specificity of 100% as a diagnostic test of adverse neurodevelopmental outcome (Table 2) [50].

Comporti et al. found, in a cohort of 24 preterm newborns and 27 term newborns, higher F2-isoprostanes in the blood of preterm than term infants in correlation with GA. They suggested the involvement of OSM in the physiopathological changes related to perinatal growth [129]. An identical pattern was observed when NPBI was measured in a cohort of 30 preterm and 29 term newborns [130].

Cord blood OSM were assessed in term newborn (n = 28), preterm newborn (n = 28) IUGR infants and controls (n = 24). The results showed that increased levels of peroxynitrite anion and thiobarbituric acid-reactive substances correlated with the CNS maturity of IUGR infants [131].

OSM (MDA, SOD, catalase, CAT; GSH) were assessed in the cord blood of 20 SGA term newborns and 20 controls. Significant differences were observed between the groups, suggesting that intrauterine malnutrition is associated with increased OSM in SGA term newborns [132]. Furthermore, OSM (MDA, SOD) were measured in SGA infants delivered by cesarean section (n = 21) or vaginally (n = 21). Results showed that SGA term newborns delivered by caesarean section had insufficient protective mechanisms against increased OSM at birth [133].

Higher longitudinal blood OSM (MDA, PC) were detected in PA-HIE term newborns (n = 40) than in controls (n = 40). The data correlated with the occurrence of 8 m adverse neurological outcome and early neonatal death [134].

In HT-treated PA-HIE term newborns (n = 10) and controls (n = 11), blood OSM (total hydroperoxides) were longitudinally measured up to 72 h from birth. Higher OSM were found in HT-treated term newborns, suggesting a partial protective action by HT [135].

Blood OSM (CAT, GSH, nicotinamide-adenine dinucleotide phosphate ratio, MDA) concentrations were also compared in term (n = 100) and preterm newborns (n = 100). Lower OSM were observed in preterm newborns, suggesting that they are much more exposed to OSM at birth and are susceptible to antioxidant deficiencies [136].

Finally, levels of OSM (xanthine, hydroperoxides, advanced oxidative protein products, glutathione s-transferases) measured in urine have been found to correlate with non-CNS diseases [137, 138].

GFAP

GFAP is a cytoskeletal monomeric filament protein, detectable in the astroglia of the CNS, representing a specific marker of differentiated astrocytes [139, 140]. As GFAP is not routinely secreted in blood but only as a consequence of astrocyte death, it has been investigated as a biomarker for brain injury.

CSF

In the CSF of a cohort of preterm neurologically abnormal (n = 10) or normal newborns (n = 7) and of term newborns (n = 9), increased levels of GFAP were found to correlate with abnormal neurological outcome [141].

Blood

Longitudinal GFAP cord blood levels (0–96 h) were assessed in 21 very low birthweight (VLBW) newborns, affected by PVL and white matter injury (WMI) on CUS at 6 weeks of life, matched for GA with 42 healthy controls. GFAP was higher in damaged infants from 24 to –96 h time-point. A GFAP cut-off >0.04 ng/mL at 24 h time-point achieved a specificity and a sensitivity of 91.2% and 52.4% as predictor of PVL and WMI [51] (Table 2).

In 56 CHD term newborns, blood GFAP levels at birth were superimposable on those measured in 23 controls,

and started to change after the transitional phase (>72 h) in parallel with closure of the patent ductus arteriosus [142].

GFAP cord blood levels were investigated in PA-HIE and controversial results have been observed. In particular: (i) no differences between PA-HIE newborns (n = 15) and controls (n = 31) [143]; (ii) no differences and no correlation with the outcome at 36 months follow-up [144], (iii) higher GFAP from birth to 96-h of life in PA-HIE newborns (n = 15) than in controls (n = 11) [55], and (iv) increased GFAP at birth in 20 PA-HIE term newborns developing poor neurological outcome at 18 months follow-up [52]. GFAP at a cut-off of >0.08 ng/mL at 12 h achieved a PPV and NPV of 100% and 0%, respectively [52] (Table 2).

In 23 HT-treated PA-HIE infants, GFAP was predictive of brain injury as shown by MRI patterns [145]. In 64 HT-treated PA-HIE infants, Jiang et al. found higher longitudinal GFAP in moderate/severe than in mild PA-HIE cases [54]. Finally, in 20 PA-HIE infants Massaro et al. found increased GFAP at 24–72 h in cases with poor/ominuous outcome. In light of this, GFAP at a off of 0.2 ng/mL achieved a specificity of 90%, a sensitivity of 78% and PPV-NPV of 82%–87%, respectively, as predictor of poor/ominous outcome [53] (Table 2).

More recently, Patil et al. measured GFAP in a cohort of PA infants potentially suitable for HT. They found a significant correlation between increased GFAP levels and the occurrence of abnormal aEEG [146].

UCH-L1

UCH-L1 is a neuron-specific cytoplasmatic enzyme concentrated in the perikarya and dendrites of neurons. Some authors have proposed UCH-L1 as a desirable NB as it is an abundant protein specific to the CNS and resistant to endogenous proteases [147].

CSF

To the best of our knowledge, no studies have measured UCH-L1 in the CSF of preterm and term newborns.

Blood

In blood, increased UCH-L1 levels have been reported as an expression of altered blood-brain barrier permeability [148]. Blood UCH-L1 has also been investigated as a marker of traumatic injury in pediatric patients, with promising results [148, 149].

In 15 PA infants with moderate/severe-HIE and 31 controls, cord blood UCH-L1 has been reported not to differ among studied groups [143]. Furthermore, Jiang et al. found higher UCH-L1 in 31 HT-treated PA-HIE infants than in 34 controls: UCH-L1 also correlated with the degree of HIE [54].

In 20 HT-treated PA-HIE infants, Chalak et al. found no correlation between UCH-L1 and adverse neurological outcome [52]. Conversely, Douglas-Escobar found that UCH-L1 in the cord blood of 16 PA infants was associated with cortical injury and later motor and cognitive development [55]. The same Authors, in a cohort of 14 PA-HIE infants, found a correlation between UCH-L1 and the occurrence of ominous outcome, with a specifity of 95% for a cut-off value of 28.0 ng/mL [56]. Increased UCH-L1 has also been reported by Massaro et al. at different HT monitoring time-points in 20 PA-HIE infants with poor outcome. At a cut-off of 13.8 ng/mL, UCH-L1 reached a specificity of 100% and a sensitivity of 75% as a diagnostic test for poor outcome [53] (Table 2).

More recently, Patil et al. measured UCH-L1 in a cohort of PA infants potentially suitable for HT. They found high UCH-L1 in PA infants with abnormal aEEG subjected to HT and normal levels in PA infants with normal aEEG [146].

Conclusions

Today, a reliable parameter able to predict perinatal brain damage in high-risk newborns is still eagerly awaited. NBs seem promising tools to provide useful information to front-line physicians in daily clinical practice. However, despite the large number of studies reported here, only a few NICUs include NBs in daily clinical practice. To date, no clinical protocols or guidelines regarding treatment in the perinatal period have been approved. Conversely, several biomarkers (S100B, GFAP, UCH-L1) have recently been approved by European and US health care agencies [149] for use with adults and children, especially in cases of traumatic brain injury [150].

The fact that biomarkers are not included among standard monitoring procedures in the perinatal period, even for the early detection of short-/long-term brain damage, warrants further consideration. To the best of our knowledge, there are several cons that need to be taken into due account. Briefly, the main limitations are:

1. the design of the trials themselves in terms of small cohort sizes, lack of multicenter investigations, heterogeneity of the neurological complications investigated, and lack of stratification according to the severity of the main perinatal diseases (IUGR, IVH, PVL, WMI, PA-HIE);
2. the lack of conclusive data showing that NBs could support standard monitoring parameters in the early identification of cases that do or do not require HT treatment;
3. the different techniques for assessing NBs (i.e. ELISA, electrochemiluminescence immunoassay, HPLC) in terms of reproducibility, sensitivity, specificity and optimal timing for obtaining results, each of which may provide different results for a single patient or sample. The point is crucial for the accuracy of patients' diagnoses and the identification of those suitable for a specific treatment at the appropriate time;
4. the lack of consensus among manufacturers for the validation of non-invasive methods of performing assays in biological fluids such as urine and saliva that constitute the best option for less stressful longitudinal brain monitoring,
5. the lack of reference curves for the period of investigation for each biomarker, technique and biological fluid.

The solution thus lies in multidisciplinary cooperation among neonatologists, pediatricians, biochemists, neuroscientists and manufacturers. Once such a multidisciplinary team can clarify these points, we believe it should be possible to answer the question as to what more is needed. Some authors have suggested that a panel of multiple biomarkers, rather than a single one, could provide additional accuracy in the early assessment of cases at risk for perinatal brain damage. This is especially true for PA: bearing in mind the timing of the cascade of events that leads to brain injury, the possibility that a panel of biomarkers could provide useful information in the delicate post-insult phases is more than a mere hypothesis. Studies in both animal models and humans have shown that neuro-proteins, calcium-binding proteins, vasoactive agents and oxidative stress markers are all involved, at different times, in the known phases of pathophysiological PA leading to brain damage. The value of additional biomarkers may lie in their potential to provide useful information for the validation of future therapeutic strategies. In this regard the possibility of a complex therapeutic protocol able to support neuro-protection through multiple treatments at different time-points and for different lengths of administration has been suggested for the first 96 h from an insult. Studies are in progress and the results are eagerly awaited.

We are not claiming that any one marker is of major clinical significance; we are reporting the state of the art regarding the principal biomarkers whose use in the perinatal period is discussed in recent FDA, EMA and NIH statements (Table 1). We are of course aware that there is increasing knowledge concerning new biomarkers such as Tau protein, spectrin breakdown products and inflammatory cytokines (i.e. IL 6, etc.). Despite the promising results, further investigations are needed before they can be considered to meet international health agencies' requirements.

Table 3 shows a detailed description of biomarkers and their fulfillment of FDA and EMA criteria. The findings identify differences in laboratory performance that need

Table 3: Optimality items for an NB according to the FDA and the EMA criteria: marker of brain damage and of degree and extension of the lesion; possibility of longitudinal monitoring; studied in pediatric/neonatal populations and provided of reference curves; measurable by commercial kits; measurable in different biological fluids.

NB	Brain damage	Degree of injury	Lesion extension	Longitudinal monitoring	Pediatric population	Available kit	Reference curve	Biological fluid	Results output, h
S100B	Y	Y	Y	Y	Y	Y	Y[a]	CSF, AF, CB, PB, U, S, M	<1
AM	Y	N	N	Y	N	Y	N	CSF, A, C, P	<2
AcA	Y	N	N	Y	N	Y	N	CSF, A, C, P, U, M	<2
NSE	Y	Y	N	Y	N	Y	N	CSF, C, P	<2
OSM	Y	Y	N	N	N	Y	N	CSF, C, P	<2
GFAP	Y	N	N	Y	N	Y	N	CSF, C, P	<2
UCH-L1	Y	Y	Y	Y	N	Y	N	C, P	<2

AM, adrenomedullin; AcA, activin A; NSE, neuron specific enolase; OSM, oxidative stress markers; G-FAP, glial fibrillary acid protein; UCH-L1, ubiquitin carboxyl-terminal hydrolase L1; Y, yes; N, no; CSF, cerebrospinal fluid; AF, amniotic fluid; CB, cord blood; NB, neurobiomarkers; PB, peripheral blood; U, urine; S, saliva; M, milk. A, Waiting for studies in wider healthy populations.

further investigation. One major point regards the possibility of using biomarkers for assessment in different biological fluids. Today, S100B protein is the only biomarker that has been reported to be detectable in both urine and saliva whilst no data have been reported regarding the use of other markers in fluids, except AcA, which has been measured in urine. Another crucial issue is reproducibility, as is the time required to obtain the results. Bearing in mind the known pathophysiological steps leading to perinatal brain damage, results need to be obtained as early as possible. The ELISA and HPLC techniques can currently provide results within 2–6 h, whilst the electrochemiluminescence immunoassay offers S100B results within 2 h (median 45 min).

Last but not least, the costs of sampling are another relevant point: to the best of our knowledge there are no significant differences among different techniques. It is noteworthy that the cost/benefit of each biochemical marker is lower than that of any of the standard monitoring procedures currently used for monitoring the brains of sick newborns and children.

Future prospects

Recent advances in laboratory technology and performance suggest that, despite the limitations mentioned, we are not so far from reaching the target: the inclusion of biomarkers in clinical guidelines for perinatal patients in the same way that they are for adult and pediatric patients. Among new diagnostic tools metabolomics appears to offer a highly promising research field and interesting preliminary results have been reported in high-risk newborns [151]. However, further investigations are needed in wider populations before this approach can start to fulfill FDA, EMA and NIH criteria and be included in clinical guidelines.

The new challenge for the multidisciplinary team will regard the improvement of techniques for the measurement of biomarkers and the choice of biological fluid for assessment. The former will involve, at the very least: (i) identification of the sample volume required for the measurement of biomarkers, and (ii) the time required for results to be ready, in order to be able to select the cases suitable for treatment and start therapeutic strategies with the least possible delay. In addition, the possibility to measure a panel of biomarkers simultaneously, especially during different PA phases, is highly desirable.

The latter issue is an especially interesting avenue of investigation. Ideally, all biomarkers suitable for inclusion in clinical guidelines should be measurable in biological fluids that can be collected using non-invasive techniques, such as urine and saliva, in order to ensure that longitudinal monitoring of the brains of sick newborns is as accurate and useful as possible. However, bearing in mind that the principal goal of perinatologists is prevention, additional data on the assessment of S100B and other biomarkers in maternal blood in high-risk pregnancies will remain a key area of investigation aimed at determining the timing of insults and the relevant treatments as early as possible. Nonetheless, the assessment of biomarkers in maternal blood will offer physicians useful information on fetal-maternal well-being and on what is currently considered the prime objective of obstetricians: the optimal timing of delivery.

Finally, as the usefulness of biomarkers in evaluating the effectiveness/side-effects on the CNS of therapeutic strategies has been shown, it is reasonable to suggest that the longitudinal assessment of these proteins be adopted for monitoring fetal/neonatal well being in both healthy and high-risk cases [152].

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