www.jpnim.com Open Access eISSN: 2281-0692
Journal of Pediatric and Neonatal Individualized Medicine 2013;2(2):e020203
doi: 10.7363/020203 Received: 2013 Apr 11; accepted: 2013 Jun 12; advance publication: 2013 Jul 03

Review

Inflammation and oxidative stress biomarkers in neonatal brain hypoxia and prediction of adverse neurological outcome: a review

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Abstract

Despite advances in perinatal care, the outcome of newborns with hypoxic-ischemic encephalopathy is poor and the issue still remains challenging in neonatology. The use of an easily approachable and practical biomarker not only could identify neonates with severe brain damage and subsequent adverse outcome, but could also target the group of infants that would benefit from a neuroprotective intervention. Recent studies have suggested interleukin-1b, interleukin-6, tumour necrosis alpha (TNF-a) and neuron specific enolase (NSE) to be potential biomarkers of brain damage in asphyxiated newborns. S100B, lactate dehydrogenase, nitrated albumin-nitrotyrosine, adrenomedullin, activin-A, non protein bound iron, isoprostanes, vascular endothelial growth factor and metalloproteinases have also been proposed by single-centre studies to play a similar role in the field. With this review we aim to provide an overview of existing data in the literature regarding biomarkers for neonatal brain damage.

Keywords

Brain damage, inflammation, oxidative stress, biomarkers, neonatal asphyxia, neurological outcome.

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How to cite

Varsami M, Xanthos T, Aroni F, Argyri I, Lelovas P, Dontas I, Papalois A, Fanos V, Iacovidou N. Inflammation and oxidative stress biomarkers in neonatal brain hypoxia and prediction of adverse neurological outcome: a review. J Pediatr Neonat Individual Med. 2013;2(2):e020203. doi: 10.7363/020203.

Introduction

Despite advances in perinatal care over the last decades, hypoxic-ischemic encephalopathy (HIE) remains a major cause of morbidity and mortality in the neonate, leading to neurodevelopmental deficits in childhood. The prevalence of HIE varies among different countries, it is however estimated that it affects 0.1-0.5% of live births, accounting for 23% of neonatal deaths worldwide [1].

The degree of brain injury is the major determinant of the final outcome. The severity of HIE is usually classified as mild, moderate or severe [2]. The main cause of perinatal asphyxia is the interruption in placental blood flow leading to brain cell ischemia-anoxia triggering anaerobic glycolysis. This in turn results in high consumption of ATP reserves, accumulation of lactic acid and failure of trans-cellular ion pumps with subsequent accumulation of Ca^{+2}, cytotoxic edema and release of neurotransmitters [3, 4]. At the same time, the combination of activation of protein synthases secondary to increased intracellular Ca^{+2}, reduction of electron transport chain components, release of iron from ferritin and increased degradation of ATP, leads to formation of free radicals, causing oxidative stress and neuronal death [5]. In the post-resuscitation period, even if cerebral blood flow and oxygen delivery to the brain are restored, there is a second phase of brain injury, mostly attributed to activation of apoptotic mechanisms [6]. Poor neurodevelopmental outcomes may be primarily attributed to this delayed phase of on-going brain injury, estimated to evolve within 6-72 hours post-hypoxia [3, 4, 7]. This time period has been so far the targeted therapeutic window for the implementation of neuroprotective methods, such as whole body cooling [8].

Taking into consideration that any intervention has to be implemented as soon as possible after the hypoxic-ischemic insult, it is vital to determine the group of newborns at high risk that will benefit more from these interventions. So far, this has been mostly based on a combination of clinical variables, including labour complications, a low 5 min-Apgar score, need for resuscitation and fetal acidemia [9]. Early assessment of cerebral function can be made by a combination of an abnormal early neurologic examination with abnormal electroencephalogram (EEG). However, not all babies with subsequent HIE will develop seizures or abnormal neurological findings within the first hours after birth [10]. The predictive value of an abnormal EEG is even higher when combined with abnormal neuro-imaging findings on magnetic resonance imaging (MRI) [11]. However these findings are not evident within the first 48 hours, except perhaps for diffuse brain edema [12]. More advanced MR techniques, such as diffusion-weighted imaging and magnetic resonance spectroscopy (MRS) may detect brain damage in even earlier stages [13, 14]. Not all neonatal units, though, have access to these specialised neuroimaging techniques.

Unfortunately, even when combining clinical signs and these techniques, early identification of neonates, who may subsequently have poor neurodevelopmental outcome, can still be challenging. In this context, the use of a biomarker of inflammation or oxidative stress that will increase within the first hours of life in these neonates may help in the early diagnosis of HIE and promptly identify neonates who may qualify for neuroprotection. Despite extensive research in the field over the last few years, no such biomarker has been available in clinical practice so far. The poor generalizability of previous biomarker studies may be attributed to variations across studies in regards to the following issues: inclusion criteria, follow-up periods and sample time, ranging from 1 hour to 3 or 4 days.

With this article we propose to review data in literature on potential biomarkers of brain damage that have been used in experimental models and clinical studies, with a primary focus on the time of sampling of the biomarker, set cut-off values and specificity in terms of prognosis. The use of the biomarkers; cytokines, neuron specific enolase and S100B, adrenomedullin, nitrotyrosine-nitrated albumin, lactate dehydrogenase, activin A, non-protein bound iron, isoprostanes, vascular endothelial growth factor and matrix metalloproteinases, will be examined in depth.

Cytokines

Cytokines are molecules with a wide range of action participating mostly in growth, development and inflammation [15]. During, or post-hypoxia, a local inflammatory response stimulates the blood vessel endothelium to produce cytokines [16, 17]. Experimental and clinical research in adult brain injury has confirmed that the levels of pro-inflammatory cytokines increase after an ischemic insult [18]. Nonetheless, their role in the underlying pathophysiology has not been fully elucidated. Animal experimental models of perinatal asphyxia have demonstrated that IL-1b and IL-6 increase after hypoxia-ischemia, peaking at 4-6 hours and returning to normal levels by 24 hours. Moreover, pre-treatment with an IL-1b receptor antagonist reduced hypoxic

ischemic brain damage [19, 20]. Furthermore, neuronal apoptosis in asphyxiated rats was positively correlated with increased levels of TNF-a, ICAM-1 and IL-1b by 6-72 hours after the hypoxic insult [21]. In addition, IL-6 levels in cerebrospinal fluid (CSF) obtained 12-72 hours after birth not only were significantly higher in asphyxiated newborns with stage III HIE, but also correlated with adverse outcome at 3 months of age [22]. In line with these findings, Savman et al, reported that among asphyxiated infants, high CSF IL-6 and IL-8 levels obtained within 72 hours from birth correlated to the degree of neonatal encephalopathy and adverse outcome [23]. Chiesa et al investigated the association between maternal and cord IL-6 levels and degree of encephalopathy or outcome. Even though at the time of delivery mothers of asphyxiated infants had higher levels, no correlation with HIE or outcome was found. On the other hand, cord IL-6 levels correlated to the degree of HIE and to adverse outcome at 2 years of age [24]. Serum IL-6 levels, alone or in combination with imaging, have also been related to HIE and poor outcome [25-29]. The CC genotype of IL-6 was recently reported to be more common in asphyxiated newborns and correlated to abnormal neurological outcome when on the contrary the CG genotype was found to be correlated with favourable outcome [30].

Serum, CSF and cord levels of pro-inflammatory IL-1b have also been investigated. CSF IL-1b levels (obtained 4-24 hours post-asphyxia) correlated with the degree of HIE and adverse outcome, compared to those with normal neurodevelopmental assessment at 1 year of age [31]. Umbilical cord IL-1b was also proposed as a potential biomarker of brain damage, as its levels were significantly higher in neonates with HIE and predictive of severe HIE and abnormal outcome at 6-12 months of age [32]. Several other pro- or anti- inflammatory cytokines have been implicated in the pathophysiology of HIE. In an animal model of perinatal asphyxia, IL-18 mRNA and protein increased from day 1 to day 14 after brain injury. IL-18 deficient animals exhibited decreased brain injury, thus suggesting a role of IL-18 in the pathophysiology of perinatal brain injury [33]. IL-10 and IL-8 were also elevated in asphyxiated neonates, but no correlation with the severity of HIE or outcome was demonstrated [34, 35]. Regarding TNF-a, results among studies were very heterogeneous. A meta-analysis did not reveal any association between TNF-a and outcome, while both serum and CSF IL-1b and IL-6 were predictive of abnormal outcome. Most of the studies included in this meta-analysis did not suggest a cut-off value and the time of sampling and time-frame of neurodevelopmental follow-up varied widely [36]. A recent clinical study showed that increased levels of cytokines were correlated with seizures secondary to HIE; more specifically, most cytokines increased within the first 24 hours and subsequently decreased after 72 hours except for IL-8 and IL-10 which remained elevated [37]. Chemokine CCL2 (also known as MCP-1) was found *in vitro* to inhibit cytokine production as CCL2-deficient neonatal rat cells exhibited significantly increased expression of IL-6 mRNA [38]. Galectin-3 showed increased gene and protein expression in activated microglia/macrophages of newborn asphyxiated rats, whereas galectin-3 deficient mice were protected from brain injury [39].

Neuron Specific Enolase and S100B

Neuron specific enolase (NSE) and S100B protein are commonly referred to in the literature as "brain specific proteins". Enolase is a glycolytic enzyme that catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate. Neuron-specific enolase is the dominant enolase isoenzyme found in neurons, in neuroendocrine cells and in neuroendocrine tumors and is released in CSF and blood after brain injury. So far, it has been extensively investigated and used as a diagnostic marker for neuroblastoma and small cell carcinoma [40]. S100 proteins, on the other hand, consist of a group of calcium-binding proteins located in astroglial as well as in other neuronic cells and are mainly excreted in urine. More than 20 S100 proteins have been so far identified and are implicated in psoriasis, rheumatoid arthritis, cystic fibrosis, cardiomyopathy, multiple sclerosis, Down's syndrome, Alzheimer's disease and many tumors. S100 proteins are found in the brain as homodimers or heterodimers of 2 subunits; S100A is present in high concentrations in glial and Schwann cells, whereas S100A is present in glial but not in Schwann cells. CSF and serum concentrations of S100B and NSE were reported in adults to be diagnostic of cerebral damage (i.e. traumatic brain injury, stroke, subarachnoid hemorrhage) and reflective of the clinical course and severity of the disease [41]. In healthy children, S100B is measurable within the first 3 years of life and was moderately inversely correlated with age [42]. Serum and CSF S100 correlated with brain damage in children with traumatic brain injury and in preterm neonates with intraventricular hemorrhage (IVH) [43-45]. Women in preterm labour, with intact membranes and intramniotic infection have also been reported to have significantly higher S100B levels in amniotic fluid [46]. Increased serum S100 levels were reported

to be higher in asphyxiated neonates compared to control groups and to be progressively decreasing from day 1 to day 4 or 7; however, they were not related to long-term neurodevelopmental outcome [45, 47] Gazzolo in 2004 indicated that urinary S100B in the asphyxiated group was significantly higher at all time-points monitored and that it was significantly higher in the group with moderate or severe HIE compared to that with mild HIE. Possible correlation of S100B to subsequent neurodevelopmental outcome was not, however, examined in this study. [46] Urinary S100B measured soon after birth (defined as first urination in the study) had a 100% sensitivity and specificity, with a cut-off value > 1 μg/l, as a single marker for predicting ominous outcome, i.e. neonatal death within 7 postnatal days [48]. Renal impairment or failure, which almost inevitably accompanies HIE, was not a confounding factor in urinary S100B levels measurements, therefore increasing its diagnostic significance as brain specific marker [49].

The first studies that correlated NSE levels with HIE and poor outcome were first published in the mid 1990s. Garcia-Alix and co-workers associated increased levels of CSF NSE at 12 and 72 hours of life with the severity of encephalopathy and adverse outcome at 1 year of life [50]. Thornberg et al similarly reported that NSE levels were significantly more elevated in the CSF of asphyxiated term newborns with HIE (median value 25.4 μg/l) compared to the control group (median value 10 μg/l, $p < 0.001$). CSF was taken at 2-32 hours of life and in individuals in whom concomitant serum sample was available. It was shown that serum values roughly doubled in those with HIE [51]. This correlated with subsequent studies that suggested a serum NSE cut-off value of 40 μg/l for neonates with moderate or severe HIE, obtained within the first 48 hours of life [52]. Regarding prediction of outcome, NSE was found to be significantly higher in asphyxiated newborns with adverse neurological outcome or death (mean: 116.4 ng/ml), in comparison to those with favourable outcome (mean: 21.3 ng/ml) [53]. Several other studies also indicated that serum NSE could be predictive of the degree of HIE and poor outcome, but the timing of the blood sampling among those studies ranged from 3 hours to 7 days of life [26, 45, 54]. According to a recent meta-analysis, although most of these results showed a positive association with the degree of HIE and/or with poor neurologic outcome, CSF NSE was found to be associated with poor neurologic outcome [36]. However, few studies published later were not included in this meta-analysis, i.e. serum NSE [45, 54], urinary S100B [55, 56].

Adrenomedullin

Adrenomedullin is a 52-amino acid vasodilating peptide, expressed mainly in endothelial cells with a wide range of other roles in apoptosis, angiogenesis and growth [57, 58]. Jensen et al in an animal model suggested that adrenomedullin mRNA expression increased in the cerebral cortex of asphyxiated foetuses [59]. Trollmann et al demonstrated that placental gene expression of adrenomedullin mRNA was significantly higher in asphyxiated newborns with moderate or severe HIE compared to those with mild HIE. Hypoxia also increased adrenomedullin mRNA expression by human leucocytes at 4 hours after birth [60]. Asphyxiated neonates who subsequently developed intraventricular hemorrhage (IVH), had significantly elevated adrenomedullin levels at 12 hours after birth, compared to asphyxiated infants without IVH [61]. These results support the hypothesis that adrenomedullin expression is increased in response to hypoxia and may indicate newborns with hypoxic brain damage. It still remains unclear whether increased levels could be directly attributed to cerebrovascular damage *per se* or could be attributed to the associated pathology that led to the hypoxic event.

Nitrotyrosine - Nitrated albumin

Large amounts of nitric oxide (NO) are produced during hypoxia-ischemia and reperfusion. Its reaction with superoxide ions leads to the production of highly toxic reactive species. As it is practically impossible to measure the concentrations of these species, their reaction products and metabolites have recently been studied as markers of oxidative stress. Nitrotyrosine is a product of tyrosine nitration, mediated by reactive nitrogen species and is considered a marker of oxidative stress. It has been studied so far as such a marker in numerous neurologic and non-neurologic conditions in adults and it has been related to brain tissue damage. Serum nitrotyrosine in a newborn rat experimental model increased at 30 minutes after hypoxia-ischemia induced by carotid occlusion [62]. Groenendaal et al reported that nitrotyrosine was present in brain tissue (mainly in the thalamus, base and motor nuclei of the pons and inferior olives) of 22 full-term infants, as well as in the spinal cord of 5 out of 18 full-term infants who died after severe perinatal hypoxia [63, 64]. A multicenter study investigated the significance of nitrated plasma albumin concentrations in correlation with the grade of neonatal encephalopathy (NE). Nitrated albumin was significantly increased in

newborns with moderate or severe NE compared to controls or those with normal mild NE on day 1 [65]. The fact, however, that D0 and D4 nitrated albumin levels were not significantly different among groups limits its value as a biomarker useful for therapeutic intervention within the first hours of life.

Lactate dehydrogenase

Clinical practice and clinical and experimental studies have shown that serum levels of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in asphyxiated newborns increase within the first hours of life [66, 67]. A multi-center study investigated whether the levels of the above enzymes could be related to the degree of HIE and long-term adverse neurodevelopmental outcome. Levels of these three enzymes were significantly increased in newborns with moderate to severe encephalopathy compared to normal or newborns with mild HIE. LDH predicted more reliably adverse neurodevelopmental outcome at 18 months of age, even though the positive predictive value was lower for the prediction of HIE. Among these three enzymes, LDH had the highest sensitivity (100%), specificity (98%) and predictive value (90%) for moderate to severe HIE [68].

Activin A

Activin A is a protein complex that belongs to the TGF-β family that was initially reported to stimulate the production and excretion of FSH. In addition, activin A was reported to be produced by several organs including specific regions of the brain (olfactory bulb, striatum, pons/medulla, hypothalamus) [69]. Although there is limited knowledge on the exact function of activin A in neuronal development, animal models have shown that cerebral hypoxia and ischemia increased the expression of activin A mRNA [70, 71]. Additional experimental data support the role of neuronal excitatory upregulation in its expression [72]. Since it is produced in the placenta, activin A increases during pregnancy and is associated with pre-eclampsia and intrauterine growth restriction (IUGR) [73, 74]. Florio et al reported that in asphyxiated newborns, CSF levels in the first 24 hours of life were significantly higher in those neonates who subsequently developed moderate or severe HIE [75]. In addition, urinary activin A may be used as a potential biomarker of perinatal brain damage, since concentrations of activin A from urine collected at birth and within the first 72 hours were significantly raised in asphyxiated newborns with severe HIE [76]. More recently, serum levels of Activin A, taken at 12, 24 and 72 hours after birth were also correlated with the occurrence and the severity of HIE [77]. Furthermore, Fiala et al also demonstrated that umbilical cord Activin A was significantly higher in hypoxic compared to non-hypoxic infants whereas there was no association with subsequent occurrence of IVH [78]. Nonetheless, existing literature is controversial since increased umbilical cord levels were found to be correlated with advancing gestational age and mode of delivery; they were not associated, however, with cord pH and thus, hypoxia [79, 80]. This ambiguity could be explained by the fact that activin A is expressed by many organs, including placenta, is triggered by several factors and has a wide range of actions, not clearly defined yet; thus its increase could be attributed not only to feto-placental hypoxia-ischemia but also to concomitant or underlying pathology.

Non-protein bound iron

Brain tissue is rich in polyunsaturated fatty acids which are highly susceptible to oxidative reactions and are increased by hypoxia, ischemia, a relatively hyperoxic environment, release of glutamate and free iron [81, 82]. Magnetic resonance studies in perinatal asphyxia animal models have substantiated *in vivo* the above findings during the acute phase of hypoxic insult and brain reperfusion [83, 84].

Iron induces activation of reactive oxygen species. Animal models have shown that iron content, as well as the expression of iron regulating proteins, significantly increase in hypoxic brain cells [85, 86]. In hypoxic newborns, iron release from erythrocytes increases after birth and indicates long-term adverse neurological outcome, while it is correlated with high levels of non-protein bound iron (NPBI) [87, 88]. NPBI is a pro-oxidant that converts hydrogen peroxide into the highly toxic hydroxyl radical and is used as an indirect marker of free radical release. Studies indicate that levels of NPBI significantly increased within 24 hours after birth in asphyxiated compared to non-asphyxiated newborns [89]. In addition, NPBI plasma levels at 0-8 hours after birth were significantly higher in severely asphyxiated neonates compared to healthy controls. These levels were significantly correlated to neurodevelopmental outcome at 1 year of age [90]. Buonocore et al, in a study that enrolled 384 term and preterm newborns, reported that asphyxiated neonates with subsequent abnormal neurodevelopmental outcome at 2 years of age had significantly higher cord blood levels of hypoxanthine, nucleated red

blood cell count, non-protein bound iron (NPBI) and advanced oxidative protein products. NPBI is the most significant predictive marker for neurodevelopmental outcome [91].

Isoprostanes

Isoprostanes are prostaglandin-like compounds that are formed by free radical peroxidation of arachidonic acid, while neuroprostanes are isoprostane-like compounds that are formed by free radical mediated oxidation of docosahexaenoic acid. Isoprostanes are stable molecules, easily detectable by sensitive methods and are reliable markers of oxidative stress and lipid peroxidation [92]. In an animal model of global perinatal asphyxia, animals subjected to 20 min of asphyxia, compared to those exposed for 10 min, exhibited significantly increased F_2-isoprostane levels in the brain tissue within 2 hours of post-hypoxic insult [93]. Cord levels of total and free F_2-isoprostanes in preterm and term newborns were inversely correlated with gestational age. More specifically, cord levels of free F_2-isoprostanes were significantly higher in term and especially preterm newborns compared to adults. A positive correlation between NPBI and total and esterified isoprostanes was also found, suggesting that NPBI in plasma is primarily involved in the formation of esterified F_2-isoprostanes [94].

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a protein involved in angiogenesis. Its role in asphyxia, both in adults and newborns, is still under investigation. Animal models of perinatal asphyxia have demonstrated that VEGF mRNA expression is significantly higher in hypoxic brain tissues. In these models, VEGF expression reached its peak on day 1 and decreased by day 7-14. It was also associated with neuronal apoptosis [95-97]. Another animal model demonstrated that hypoxia significantly increased VEGF mRNA expression in placenta and brain of newborn asphyxiated animals [98]. These experimental data were also strengthened by clinical studies, which have demonstrated the association of HIE with CSF or cord blood VEGF. VEGF levels in cord blood following birth asphyxia were raised and specifically increased more in those neonates who later developed encephalopathy [99]. CSF levels of VEGF were raised in hypoxic neonates but there was no association with the different stages of HIE [100]. Contrarily, Vasilevij et al demonstrated that concentrations of VEGF in the CSF of neonates with moderate and severe HIE were higher than those with mild HIE and were also correlated with adverse neurological outcome at 12 months of age [101].

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) consist of a group of proteolytic enzymes mediating the neuroinflammatory response to hypoxia-ischemia in the developing brain [102]. Inhibition of MMPs in animal models results in a better outcome. On the other hand, given the suggested neuroprotective role of MMPs and the delicate balance of developmental mechanisms and neuroplasticity, it remains controversial whether this inhibition could be beneficial or detrimental [103-105]. In terms of brain damage detection, animal data support the increased expression of MMPs in brain tissue within the first day after the hypoxic insult [106-109]. In line with these results, clinical studies have demonstrated that serum MMP-9 concentrations in asphyxiated neonates with neurological sequele were significantly higher compared to controls or asphyxiated neonates without sequele [110, 111].

Discussion

Neonatal HIE is still one of the major causes of morbidity and mortality in the perinatal period, affecting approximately 3 *per* 1000 live births and causing severe neurological deficits in 25% of surviving infants [112]. Even though guidelines of resuscitation and intensive care protocols are generally the same globally, rates of HIE differ among centres and countries. The main concern for these infants, however, still remains the long-term outcome, ranging from normal neurodevelopmental outcome and standard quality of adult life to severe global developmental delay and death. Apart from the affected individuals and their families, HIE is very challenging and remains a source of major concern for clinicians and a heavy economic burden for societies. The effective management and early intervention for these infants depends on early identification of the condition.

This review aimed at identifying biomarkers of inflammation and oxidative stress studied so far (**Tables 1** and **2**) in HIE and their association with adverse outcome. Until now, none of them has been used routinely in everyday clinical practice. Apart from the biomarkers mentioned above, some others have been suggested as such, in limited studies or experimental models. Glial fibrillary acidic protein

(GFAP) is the main cytoskeleton protein in mature astrocytes and is implicated in various neurological disorders as well as in IVH in preterms [113, 114]. It reached high concentrations in the CSF of asphyxiated newborns [115, 116] and cooled neonates with HIE and abnormal brain imaging had higher serum GFAP levels compared to those with normal imaging. [117] Expression of brain derived neurotrophic factor (BDNF) was higher in animal tissue extracts from global perinatal asphyxia [118, 119] as well as in the umbilical cord and serum of encephalopathic neonates after perinatal asphyxia [120, 121]. CSF myelin basic protein [50], serum Amyloid A protein [122], serum IGF-1 [123], umbilical nucleated red blood cells in combination with lactate or early EEG [124, 125], phosphorylated axonal neurofilament heavy chain protein (pNF-H) and Ubiquitin L-terminal hydrolase (UCHL1) [126] have been suggested as early predictors of neonatal encephalopathy as well. The consistently increasing number of preterm newborns has intrigued studies of biomarkers of preterm brain injury including erythropoietin, chemokine ligand 18 (CCL18), CSF uric acid and brain-type creatinine phosphokinase (CPK-BB), transforming growth factor beta1 (TGF-β1) and beta2 (TGF-β2), plasminogen activator inhibitor1 (PAI-1), median neurofilament protein (NFL), glial fibrillary acidic protein (GFAP) and human beta-amyloid precursor protein (β-APP). [127] Even though the mechanisms of premature brain injury and HIE may not be identical, there are still underlying similarities, hence the above named biomarkers could be of value in monitoring HIE brain damage as well.

The heterogeneity and limitations of studies published so far is worth noting. First, most of the studies had a relatively small number of participating infants, while only very few were multi-center. Almost all studies included term infants, since prematurity *per se* may increase the levels of these biomarkers. Regarding inclusion criteria, however, the use of term "infants with signs of perinatal asphyxia" is vague and does not always necessarily include neonates with similar characteristics. Even though most of the studies used Apgar scores, cord pH, base excess, pathological cardiotocograph and need for resuscitation as signs of fetal distress, the cut-off values varied widely, i.e. pH less than 7.3, 7.2 or 7.0, 5-minute Apgar score < 3 or 5 and base excess > -12 or > -15. It is also worth noting that even though the need for resuscitation > 3-5 min, which reflects the condition in which the baby was born, may indicate acute or chronic perinatal asphyxia, it may not be objective in all cases. Potential biomarkers were initially studied and correlated only with the presence or absence of perinatal asphyxia and with the degree of HIE. A potential biomarker should be correlated with long-term prognosis as well, as it would be helpful for the clinicians in terms of management and implementation of neuroprotective methods. The follow-up period for each study was not the same, ranging from several weeks up to 2 years, whereas in some of the studies not all surviving infants were followed-up at the end. The definition of adverse neurological outcome either varied among studies, or was not clearly clarified or it was defined as death in some cases. Moreover, only very few studies supplied cut-off values (**Table 3**) and even among these few studies the value range was not always mentioned or the unit measurements were different, therefore making it difficult to compare but also to establish their usefulness in clinical practice.

In order to use a biomarker in clinical practice, several criteria need to be fulfilled. A potential biomarker should be:

1. Easily obtainable, rapid, not very expensive and able to be used in the majority of labour wards/hospitals. Even though CSF markers have been suggested as more specific and sensitive, the use of serum, urine or umbilical cord samples seem to be more applicable. Additionally, an easy biochemistry test, i.e. LDH, sounds more promising and practical compared to other complicated tests that could be measured only in specialised laboratories.
2. Specific for brain injury. Most biomarkers are produced not only in the brain but in many other organs as well and they can be released in a variety of other situations such as infection, prematurity, IUGR, intracerebral hemorrhage, pre-eclampsia and placental pathology. Some of them could also be attributed not only to the secondary brain injury but to the acute phase of asphyxia itself. Since in a neonate with HIE co-morbitidies may exist, it is crucial for the biomarker in question to identify those with higher risk of adverse neurological outcome due to HIE rather than other causes.
3. Increasing within the first hours of life. According to experimental data, there is a time-limited window (approximately up to 6-8 hrs post-hypoxia), during which a neuroprotective therapeutic intervention should be implemented for a more favorable outcome. Unfortunately, some of the markers that seem to be associated with brain damage increase later, i.e. at 48 or 72 hours of life.

In conclusion, there is an on-going effort in clinical research to potentially establish the clinical

Table 1. Summary of available data in the literature on brain biomarkers.

Study	Biomarker	Biological fluid	Study population	Sampling timing	P value (HIE vs mild HIE/controls)	P value (normal vs adverse outcome)	Long-term follow-up
Martin-Ancel et al., 1997 [22]	IL-6	CSF, serum	n = 20	12-72 hours	$p < 0.01$	$p < 0.05$	duration not specified
Oygur et al., 1998 [31]	IL-1b, TNF-a	CSF, serum	n = 30	4-24 hours	not studied	CSF p = 0.02	12 months
Savman et al.,1998 [23]	IL-6, IL-8	CSF, serum	n = 27	0-7 hours	p = 0.01	p = 0.05	6-48 months
Chiesa et al., 2003 [24]	IL-6	umbilical cord	n = 163	birth, 24 hours, 48 hours	$p < 0.001$	p = 0.005	24 months
Silveira et al., 2003 [25]	IL-6, TNF-a	CSF, serum	n = 35	2-26 hours	$p < 0.00001$		no
Bartha et al., 2004 [28]	IL-1b, IL-6, IL-8	serum	n = 62	1-8 days	not studied	$p < 0.005$	30 months
Tekgul et al., 2004 [26]	IL-6, NSE	CSF, serum	n = 21	24-72 hours	IL-6 $p < 0.001$, NSE $p < 0.005$	IL-6 $p < 0.001$, NSE $p < 0.005$	24 months
Fotopoulos et al., 2005 [34]	IL-8	serum	n = 106	0-24 hours	p = 0.01		no
Aly et al., 2006 [27]	IL-1b, IL-6, TNF-a	CSF, serum	n = 37	0-24 hours	IL-1b, IL-6, TNF-a $p < 0.005$	IL-1b, IL-6, TNF-a $p < 0.005$	6 and 12 months
Liu et al., 2010 [32]	IL-1b,IL-8,TNF-a	cord, serum	unknown	1, 3, 7 days			6 and 12 months
Youn et al., 2012 [37]	IL-1, IL-2, IL-3, IL-6, IL-8, IL-10, TNF-a	serum	n = 28	0-24 hours, 48-72 hours, 5 days	$p < 0.05$		6 months
Garcia Alix et al.,1994 [50]	NSE	CSF	n = 69	12-72 hours			12 months
Thornberg et al.,1995 [51]	NSE	CSF, serum	n = 22	2-64 hours	$p < 0.001$		18 months
Verdu-Perez et al., 2001 [53]	NSE	serum	n = 46	24-72 hours		$p < 0.001$	1-6 years
Celtik et al., 2004 [52]	NSE	serum	n = 102	4-48 hours, 5-7 days	$p < 0.005$		no
Gazzolo et al., 2004 [46]	S100B	urine	n = 112	0-72 hours	$p < 0.001$		no
Bashir et al., 2009 [55]	S100A1B, S100BB	urine	n = 105	0-72 hours	$p < 0.001$		no
Dai et al., 2009 [54]	NSE	serum	n = 88	12-24 hours, 7-10 days	$p < 0.01$		6 months-3 years
Gazzolo et al., 2009 [48]	S100B	urine	n = 132	0-96 hours	$p < 0.0001$		no
Distefano et al., 2009 [45]	NSE, S100	serum	n = 45	3, 24,48 hours, 7 days	$p < 0.05$		no
Liu et al., 2010 [56]	S100B	urine	n = 103	1-3 days			6 months
Trollmann et al., 2002 [60]	Adrenomedullin	placenta	n = 22	0-12 hours	$p < 0.001$		no
Di Iorio et al., 2004 [61]	Adrenomedullin	serum	n = 80	12 hours	$p < 0.001$		no
Florio et al., 2004 [75]	Activin A	CSF	n = 77	0-24 hours	$p < 0.001$		no
Florio et al., 2007 [76]	Activin A	urine	n = 60	1st urination, 12-72 hours	$p < 0.0001$		no
Florio et al., 2010 [77]	Activin A	serum	n = 105	0-72 hours	$p < 0.001$		no
Fiala et al., 2012 [78]	Activin A	umbilical cord	n = 86	birth	$p < 0.005$		no
Wayenberg et al., 2009 [65]	Nitrated albumin	serum	n = 48	1, 24 hours, 4 days	p = 0.01		no
Karlsson et al., 2010 [68]	LDH,ALT,AST	serum	n = 246	0-8 hours	$p < 0.0001$		18 months
Dorrepaal et al., 1996 [90]	NPBI	serum	n = 50	0-8 hours, 8-16 hours, 16-24 hours			12 months
Buonocore et al., 2003 [91]	NPBI	umbilical cord	n = 386	birth		$p < 0.001$	24 months
Yu et al., 2003 [89]	NPBI	serum	n = 45	0-24 hours	$p < 0.01$		5 months
Ergenekon et al., 2004 [100]	VEGF	CSF, serum	n = 32	0-24 hours	$p < 0.05$		no
Aly et al., 2009 [99]	VEGF	umbilical cord	n = 40	birth	$p < 0.005$		no
Vasilevij et al., 2011 [101]	VEGF, NSE, IL-6	CSF	n = 90	0-48 hours (IL-6), 72-120 hours (NSE, VEGF)	$p < 0.0001$	$p < 0.001$	12 months
Sunagawa et al., 2009 [111]	MMP-9	serum	n = 27	0-24 hours, 24-48 hours	$p < 0.005$		no
Bednarek et al., 2012 [110]	MMP-9	serum	n = 97	0-6 hours	$p < 0.001$	no statistical difference	not specified

Table 2. Summary of existing biomarker studies grouped according to biological fluid.

	IL-6, IL-1b, IL-8, TNF-a	NSE	S100	Activin A	NPBI	VEGF	MMP-9	Nitrated albumin	LDH, ALT, AST
Serum									
	Martin-Ancel et al., 1997 [22]	Thornberg et al.,1995 [51]	Distefano et al., 2009 [45]	Florio et al., 2010 [77]	Dorrepaal et al., 1996 [90]	Ergeenkon et al., 2004 [100]	Sunagawa et al., 2009 [111]	Wayenberg et al., 2009 [65]	Karlsson et al., 2010 [68]
	Oygur et al., 1998 [31]	Verdu-Perez et al., 2001 [53]			Yu et al., 2003 [89]		Bednarek et al., 2012 [110]		
	Savman et al.,1998 [23]	Celtik et al., 2004 [52]							
	Silveira et al., 2003 [25]	Dai et al., 2009 [54]							
	Bartha et al.,2004 [28]	Distefano et al., 2009 [45]							
	Tekgul et al., 2004 [26]								
	Fotopoulos et al., 2005 [34]								
	Aly et al., 2006 [27]								
	Liu et al., 2010 [32]								
	Youn et al., 2012 [37]								
CSF									
	Martin-Ancel et al., 1997 [22]	Garcia Alix et al.,1994 [50]		Florio et al., 2004 [75]		Ergeenkon et al., 2004 [100]			
	Oygur et al., 1998 [31]	Thornberg et al.,1995 [51]				Vasilevij et al., 2011 [101]			
	Savman et al.,1998 [23]								
	Silveira et al., 2003 [25]								
	Tekgul et al., 2004 [26]								
	Aly et al., 2006 [27]								
Umbilical cord									
	Chiesa et al., 2003 [24]			Fiala et al., 2012 [78]	Fiala et al., 2012 [78]	Aly et al., 2009 [99]			
	Liu et al., 2010 [32]								
Urine									
			Gazzolo et al., 2004 [46]						
			Bashir et al., 2009 [55]						
			Gazzolo et al., 2009 [48]						
			Liu et al., 2010 [56]						

Table 3. Cut-off values for biomarkers of brain damage by various investigators.

Study	Biomarker	Fluid	Study population	Cut-off value
Karlsson et al., 2010 [68]	LDH	serum	n = 246	LDH: 1049U/L
Aly et al., 2006 [27]	IL-1b, IL-6, TNF-a	CSF, serum	n = 37	IL-1b: 57.8 pg/ml, IL-6:10.8 pg/ml
Tekgul et al., 2004 [26]	IL-6, NSE	CSF, serrum	n = 21	IL-6:25.9 pg/ml
Celtik et al., 2004 [52]	NSE	serum	n = 102	40 µg/l
Vasilevij et al., 2011 [101]	IL-6, NSE, VEGF	CSF	n = 90	IL-6: 21.6 pg/ml, NSE: 25.5 µg/l, VEGF 196.5 pg/ml
Gazzolo et al., 2004 [46]	S100B	urine	n = 112	1.0 µg/l
Gazzolo et al., 2009 [48]	S100B	urine	n = 132	0.41 µg/l
Liu et al., 2010 [56]	S100B	urine	n = 103	0.47 µg/l
Florio et al., 2004 [75]	Activin A	CSF	n = 77	1.3 µg/l
Florio et al., 2007 [76]	Activin A	urine	n = 60	0.08 mg/l
Florio et al., 2010 [77]	Activin A	serum	n = 105	0.66 ng/ml

use of a biomarker that could identify within the first hours after birth newborns with moderate or severe HIE and associated poor neurological outcome. Although several biomarkers have been studied and suggested as such so far, there is no doubt that large multi-center studies, including long neurological follow-up, are needed in order to accept and establish the use of such a biomarker in everyday neonatal clinical practice worldwide.

Declaration of interest

The Authors declare that there is no conflict of interest.

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