Biochemical Markers for Brain Injury Monitoring in Children with or without Congenital Heart Diseases

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Abstract: Perinatal asphyxia (PA) still constitutes a common complication involving a large number of infants with or without congenital heart diseases (CHD). PA affects 0.2-0.6% of full-term neonates, 20% of which suffer mortal hypoxic-ischemic encephalopathy, and among survivors 25% exhibit permanent consequences at neuropsychological level. Each year, about one third of 1000 live births underwent to surgical intervention in early infancy and/or are at risk for ominous outcome. Advances in brain monitoring, in anesthetic and cardiothoracic surgical techniques, including selective or total body cooling, cardiopulmonary bypass (CPB) and deep hypothermic circulatory arrest, have essentially reduced mortality expanding the possibility to address functional neurologic and cardiac outcomes in long-term survivors. However, open-heart surgery constitutes a time-frame of planned ischemia-reperfusion injury, which is a price to pay in the treatment or palliation of CHD. Infants who underwent heart surgery and non-CHD infants complicated by PA share similarities in their neurodevelopmental profile and a common form of brain damage due to hypoxic–ischemic injury.

The purpose of the present review was to evaluate different mechanisms implicated in brain injury following CPB and PA and how it is possible to monitor such injury by means of available biomarkers (S100B protein, Activin A, Adrenomedullin).

Keywords: Biomarkers, brain damage, congenital disease, S100B, activin A, adrenomedullin, cardiac disease, CPB.

INTRODUCTION

Despite the continuous improvements in perinatal care, perinatal brain injury (PA), due to hypoxic-ischemic (H-I) insult, still remains the main cause of developmental disability in children [1-4]. PA incidence is around 0.2%-0.6% in term births [5], and neurological handicap affects about 50%-75% of this population of children [6]. Acute and/or chronic H-I insult may lead to long-term neurological sequelae such as cerebral palsy, mental retardation and epilepsy [7]. PA is also linked to attention deficits and hyperactivity in children and adolescents [7, 8]. The elevated incidences of some PA brain lesions [9, 10] can be at least in part due to the high H-I vulnerability of the developing brain [11-15]. To attenuate as much as possible the neurological consequences from H-I damage, the expected neuroprotective strategies include: i) quick identification of affected neonates in order to timely set the proper therapy, ii) enhancement monitoring in the perinatal period and rigorous control in intensive care, and iii) post-insult strategies to improve the evolving injury [16, 17]. The stretch of time following H-I, in order to perform successful therapeutic neuroprotection - the so called “therapeutic window” - is short (about 6 hours), thus a rapid identification of children at risk for H-I sequelae is crucial [18]. The consequence of PA damage depends on the intensity, severity and timing of the insult, as well as, particular ischemic susceptibility and immaturity of the brain. All together, the occurrence of these factors constitutes a cascade of events leading to cells death and/or state of ischemic penumbra [19-21]. Irrespective of the readjustment of normal cerebral blood flow, many of these cells die in a manner defined as delayed cell death, via process of programmed necrosis (necroptosis) and apoptosis [19-23]. These cells represent the target for therapeutic interventions in order to avoid the onset or to prevent the progression of detrimental cascades.

In CHD infants cerebral injury etiology is varied and complex. The brain damage can occurs before, during and after heart surgery. The CHD population is worthy to consider for neuroprotection studies in its own right. Approximately 30,000 infants with CHD per year belong to
United States, one third of which require surgical intervention in early infancy. Nowadays, annually about 11,000 heart operations are conducted. Advances in cardiothoracic surgical and anesthetic techniques, including cardiopulmonary bypass (CPB) and deep hypothermic circulatory arrest (DHCA), have led to a drop in mortality, extending the possibility to address functional neurologic and cardiac outcomes in long-term survivors [24-28]. Acute neuro-cardiac morbidities in infants who have CHD undergoing newborn heart surgery are well described, including seizures, stroke, choreoathetosis, and cardiac arrest. Concern in the functional condition of survivors now expands beyond the newborn period to childhood, adolescence, and adulthood [26-28]. Neurodevelopmental consequences in survivors include a distinctive profile of disturbances of cognition, behavior, attention deficit, hyperactivity disorder, executive planning, feeding, speech and language, socialization, and fine and gross motor coordination. About half of school-aged survivors of infant heart surgery receive some type of special education assistance [24-28]. In this frame, new approaches to improve neuro-cardiac outcome are obviously required. Infants who have undergone heart surgery and PA share similarities in the pathophysiological mechanisms leading to brain injury. Because PA accounts for a large percentage of chronic disability in the United States, lessons learned from preventing brain injury in infant subjected to heart surgery might be readily applicable to the larger group of non-CHD infants.

Newborn heart surgery represents a phase of programmed and intentional H-I injury, which is the price to pay in the treatment or palliation of CHD. In the past, brain injury was simply conceived as a complication of heart surgery and CPB. To date, post-surgical management is believed to have a relevant role in triggering and perpetrating brain damage.

The aim of the present review was to evaluate different cascades of events in brain injury following CPB and how it was possible to monitoring such injury using various clinical biomarkers.

**PATHOPHYSIOLOGY OF BRAIN INJURY FOLLOWING PA AND CPB**

**H-I Insult**

The developing fetal brain is highly reliant on a constant sustained blood flow and, during H-I insult, different cellular mechanisms are activated leading to cell damage [8]. The intensity, severity and timing of asphyxia, as well as peculiar ischemic susceptibility and the degree of maturity of the brain, define the size and grade of severity of the resulting damage [5, 12, 13]. There are many circumstances in which the developing brain is especially vulnerable to ischemia [2, 14, 15]. This especially holds for late preterm infants born between 34 to 36 weeks of gestation, known to be at highest risk of permanent neurological sequelae [29-32]. The explanation resides in the fact that at this stage central nervous system (CNS) growth is at the highest peak in terms of brain weight, volume, structure and function [29-32]. A tight relationship between H-I insult and the occurrence of brain damage has also been demonstrated [2, 21]. In term infants, selective neuronal necrosis, parasagittal cerebral lesion (particularly in the parieto-occipital region) and focal or multifocal cerebral lesions are the most frequent brain lesions.

In order to accomplish adequate prevention strategies become essential to improve our knowledge on the pathogenesis of brain damage. The ischemic principle is based on the role of H-I consequent of perinatal complication, often occurring in preterm and term newborns both non-CHD and CHD, caused by a breakdown of neuronal metabolism and the subsequent cerebral damage [2, 5, 33]. There is evidence that: i) a drastic and acute drop of glucose and oxygen in cerebral tissue leads to a reduced protein synthesis and neuronal death within minutes from the insult [33], ii) anoxia is a triggering factor for an excessive release of excitatory neurotransmitters, contributing to brain damage even with long-time kinesis [22], i) apoptosis could affect the consequences of ischemia when this is not severe enough to determine a tissue necrosis [22, 33]. These findings are corroborated by data on animal models in which the decline of glucose supply and the decreased availability of ATP in neuronal cells lead to a Ca++ overload, in the citoplasmatic fluids, activating lytic enzymes and reducing the realase of antioxidants and structural proteins useful for cellular homeostasis [19, 22]. One of the consequences resides in an uncontrolled release of excitatory neurotransmitters, which in turn, hyperstimulate postsynaptic neurons and oligodendroglia, through the opening of specific receptors, and leading to further incoming of calcium within cells [29]. Moreover, the production of free radicals and nitric oxide are the main factors responsible of the attack of the structural components of the neurons leading to cell damage/death [21-23]. The second consequence as a result of ischemia is cell apoptosis. It has been shown that mild/moderate ischemia is sufficient to determine the lesion of mitochondria, releasing pro-apoptotic molecules (i.e. cytochrome c) in the cytosol [32-37].

The combined effects of the aforementioned pathophysiological events act disrupting essential components of the cell with its ultimate death [2] leading to a significant increase of peculiar CNS biochemical markers such as S100B and activin A [38, 39]. On the other side, adrenomedullin (AM) is released from the extracellular space, in order to abate Ca++ release into the cytosolic compartment and the caspases activation which in turn causes apoptosis [40, 41].

**Intraoperative Events Leading to Brain Damage**

Intraoperative events are the most important causes of brain damage and include: deep hypothermic circulatory arrest (DHCA), hypoperfusion, air emboli and systemic inflammatory response.

**DHCA**

H-I injury is the main intra-operative cause of brain damage in children following open-heart surgery and CPB for CHD repair. There is now considerable evidence suggesting that the length and the degree of cooling can be deleterious on children. In this regard, it has been reported that 45 min of arrest at 18°C may be exaggerate, so that the
real ‘safe’ DHCA duration should be 20-30 min. In this time-period, the first 20 min of DHCA the cerebral metabolism still remains aerobic: thereafter, it becomes anaerobic and cerebral lactate concentrations start to increase [42]. In sheep model, after 21-33 min of DHCA, ATP cellular levels drop to the lowest point [42]. Likewise, in newborn dogs it has been observed a considerable depletion of adenine nucleotides, including ATP, in grey matter after 30 min of DHCA [43]. In a piglet model after 32 min of DHCA, phosphocreatinine and ATP become undetectable [44]. ATP depletion leads to energy failure, which in turn cause membrane ionic pump failure and depolarization. Moreover, there is an increase in free intracellular Ca$^{++}$ which activate different cellular enzymes causing neurons damage [45].

**Air Emboli**

Since most of heart-surgery procedures implicate opening the left side of the circulation, the risk that air will be forced into systemic circulation is consistent. Thanks to technological improvement in the last decades, the routine use of membrane oxygenators reduced, without eliminating, the risk of air emboli [46]. The explanations resides in the fact that: i) different oxygenators are able to remove air, ii) air forced into the venous line succeed gaseous microemboli in the arterial line, iii) perfusionist injection into the reservoir are followed by microemboli in the arterial line [47], iv) during cooling, wide temperature gradients between the pump blood and the patient increase gaseous microemboli, and, eventually, v) during rewarming, wide gradients between the heat exchanger and the pump blood cause an increase in microemboli [48]. In this setting, attention during intra-operative manoeuvres (e.g., purse string around the venous cannula, ejection of air from syringes prior to drug injection) decreases the number of cerebral emboli during CPB [49].

**Hypoperfusion**

H-I reperfusion injury may emerge during the postoperative period over several days [50]. Toxic mediators such as free radical molecules will provoke neuronal damage [51]. At a time when the energy demands of the brain have raised, oxygen delivery may be compromised due to CPB and its critical phases (cooling and rewarming). Hemodynamic patterns with are characterized by an increase in cerebrovascular resistance, elevated central venous pressure, low cardiac output and blood pressure dropping.

**Systemic Inflammatory Response**

Systemic inflammatory response activation enhances H-I reperfusion injury. In particular, the main factors involved into the systemic inflammatory response are: i) cell activation due to contact with the by-pass circuit, ii) mechanical shear stress, iii) hemodilution, hypothermia and ischemia. In pediatric CPB the response is enhanced because of a relatively greater size of the by-pass circuit due to cellular entrapment within organs, neutrophil and platelet activation, and endothelial dysfunction [52]. Complement and coagulation systems are activated and cytokines are released. Moreover, there is a wide range of variation in the release pattern and in the mediators levels in children population. A significant secretion of plasma IL-8 is observable during pediatric cardiac surgery, balanced within the plasma by a phased anti-inflammatory cytokine response [53]. There is an increase in vascular resistance, increased capillary permeability and interstitial edema. In uncomplicated cases, this response lasts not more than few days. The microcirculation of the brain is compromised by these vascular and extravascular changes. Cerebrovascular resistance increased and there is a loss of autoregulation after DHCA [54]. Heparin-bonded by-pass circuits will reduce the inflammatory response to CPB in children. Cytokines, complement, TNF and interleukin levels are decreased [55]. Heparin-bonded circuits reduced postoperative cerebral dysfunction in adults undergoing coronary artery by-pass surgery [56]. While most studies report a beneficial effect, a more recent research found out that modified ultrafiltration did not significantly influence cytokines, the complement and coagulation profiles in children undergoing cardiac surgery [55]. In pre-conditioning piglets, methylprednisolone administration four hours before by-pass significantly reduced the systemic inflammatory response and brain damage after DHCA [57]. Of note, methylprednisolone is given several hours before by-pass, since in the by-pass prime the drug administration has relatively little effect [57]. Intravenously administration of dexamethasone in children, one hour before CPB, have shown a reduction in the inflammatory response as observed by cytokine levels assessment and the clinical course [58].

**Cellular Mechanisms of Brain Injury Following CPB**

The occurrence of brain injury after HI insult is an evolving process starting during the acute insult and followed by the reperfusion phase. Identically to non-CHD infants, the main pathogenetic mechanism underlying neurological damage due to hypoxemia/ischemia, or both, is the dramatic deprivation of glucose and oxygen supply. This latter, in particular, causes a primary energy failure and activates a cascade of events eventually leading to cell damage and death [14, 59]. Of note, subsequent reperfusion injury affects brain metabolism enhancing the oxidative stress damage. The temporal patterns of glucose and energy metabolism modulation, after H-I insult, have been extensively studied and consist in primary and secondary energy failure [14, 22]. Soon after H-I, primary energy failure, depletion of oxygen precludes oxidative phosphorylation (decrease in high-energy phosphorylated compounds such as ATP and phosphocreatine), which results in a switch to anaerobic metabolism, causing release of lactate and the associated H$^+$. The accumulation of lactate and H$^+$, at a first stage beneficial for adoption to oxygen deprivation, lead to deleterious effects [2] on: i) vascular autoregulation thus advancing HI injury [22], ii) inhibition of phosphofructokinase activity by low pH [8] and, iii) the biochemical cascade leading to cellular injury [14, 22]. Secondary energy failure occurrence varies according to insult characteristics (i.e. nature and species) with onset at about 8-16 hours and a nadir at about 24-48 hours. High-energy phosphate recovering to baseline is about 2-3 hours after reperfusion and re-oxygenation, while a second decline
in high-energy phosphate is pronounced at the next 48 hours [14, 22, 60].

Primary visible feature in neuron cells is cytoplasmic vacuolation due to mitochondrial swelling, which occurs within 5–30 minutes after H-I. However, it has been observed that glucose sensitivity to oxygen deprivation is similar in differentiating oligodendrocytes as in neurons. In particular, in the immature and mature brain, the order of cellular components susceptible to H-I is neuron, oligodendroglia, astrocyte and microglia [61]. It has been demonstrated by Yue et al., that immature neurons are more prone to apoptotic neuronal death, whilst mature neurons are more sensitive to necrotic cell death [62]. The feature of neuropathology differs according to the developing age as H-I insult. The major regional patterns of selective neuronal necrosis in newborns affected by hypoxic ischemic encephalopathy (HIE), term newborns in particular, are diffuse disease, cerebral-deep nuclear with prominent involvement of cerebral neocortex, hippocampus and basal ganglia-thalamus, and deep nuclear-brain stem disease [63]. The primary form of H-I brain injury in the immature brain occurs in the cerebral white matter, leading to periventricular leucomalacia (PVL); the current available data suggest a peculiar maturation-dependent intrinsic vulnerability of premelinating oligodendrocytes (pre-OLs) to both endogenous and exogenous reactive oxygen species [63, 64]. The immature brain is particularly susceptible to ischemia in white matter because of the vascular end zones and the border zones present in this area of the brain and [2] impairment of cerebrovascular autoregulation [65]. Ness et al. have observed a transition of cell death from early necrotic deaths, to hybrid cell deaths, to classical apoptosis in white matter between 4 to 24 h of recovery from H-I [66]. This delayed pattern of apoptosis in pre-OLs, strongly supports the feasibility of intervention strategies in this specific time window to improve clinical outcomes in newborns surviving birth asphyxia.

**Experimental and Clinical Reports Supporting Brain Damage Markers Usefulness**

**S100B Protein**

As early as 1965, a neurotrophic factor was primarily discovered in bovine brain [67], and lately identified into two distinct proteins, S100β and S100α [68]. Consequent to S100 chromosomal localization in 1995, the nomenclature was modified from S100β to S100B and from S100α to S100A1 [69]. The following experimental research was therefore focused on identifying the specific role of S100B protein and protein assessment became more frequently in clinical setting.

S100 protein belongs to a family of calcium binding proteins found as homo- or hetero-dimers of two different subunits (α, β). Different combinations of the subunits make up the heterodimeric forms α-α, β-β and α-β; types are described as S100B protein and are shown to be highly specific for nervous tissue, where the protein appears to be most abundant in glial cells, although its presence it has also been reported in other neuronal subpopulations [61, 70]. S100 is a highly conserved protein in vertebrate species [71, 72], in fact the comparison of the human and bovine S100A1 and the human and rat S100B subunit reveals an almost complete homology [68].

S100B is involved in intracellular signal transduction via protein phosphorylation inhibition, enzyme activity modulation, calcium homeostasis dysregulation, and affecting cell morphology via interaction with cytoplasmatic cytoskeleton elements [73-75]. Release into the extracellular fluid, S100B acts in an autocrine and paracrine manner. S100B also demonstrated mitogenic properties in *in vitro* studies, such as the ability, at concentrations below 50 nM and above 5 μM, to increase the proliferative activity of melanoma cell lines [76], as well as on rat C6 glioma cells at 50 pM–1.5 nM [77], in particular in its oxidized state [78]. S100B dose-dependent activity, neuroprotective or neurotrophic, at different concentration it has been extensively demonstrated [79, 80]; in particular, at micromolar concentration, S100B activates the inducible nitric oxide (NO) synthase with subsequent NO generation, which potentially leads to astrocytic death [81, 82].

S100B neurotrophic properties have been provided by different studies. In fact, in cultured mesencephalic rat neurons [83] and dorsal root ganglia [84], S100B has been shown to promote neurite outgrowth [85, 86]. Moreover, it has been observed that in primary rat spinal cord culture, S100B rapidly promoted reassembly and/or stabilization of the cytoskeletal system [87], and prevented apoptosis in a neuroblastoma clonal cell line [88]. Of great importance, in newborn rats after sciatic nerve section, S100B rescued motor neuron death and preserved neuron diameter [89].

In addition to its trophic properties, a neuroprotective effect of S100B was discovered *in vitro*. S100B contrasted neuronal cell death and mitochondrial dysfunction in rat hippocampal neurons following glucose deprivation and elevated intracellular free calcium [90]. S100B is a molecule also thought to be involved in energy metabolism modulation by fructose-1,6-biphosphate aldolase [73] and phosphoglucomutase stimulation [91], and has been implicated in cytosolic Ca2+ buffering [92]. In animal cell cultures of embryonal chick and neonatal rat neurons, S100B showed the ability to preserve glutamate- and staurosporine-induced cell death [93]. However, S100B neuroprotective and neurotrophic properties still need further elucidation for what concern traumatic brain injury in particular.

Based on the aforementioned informations, S100B protein has been considered as a consolidated brain damage marker: a protein significant release has been shown one hour after H-I in rat brain slices, which furtherly enhanced after ischemic slices re-oxygenation [94]. The same pattern it has been observed in other brain injury models, thanks to the protein release by astrocytes, the main cell subpopulation responsible of S100B intracellular pool, under severe metabolic stress conditions (withdrawal of oxygen, glucose and serum in cell cultures). S100B release occurred early, and in the absence of significant cell death in the cultures, suggesting an active, stress-triggered mechanism of secretion during metabolic injury related to ischemia [95]. S100B is released into the cerebrospinal fluid (CSF) and into the blood also when structural damage occurs, as in case of infarction of glial and Schwann cells [96, 97]. A significant correlation has been reported, in human cerebrovascular diseases, between S100B plasma concentration and cerebral infarct...
volume [98]. This finding furtherly confirmed S100B putative role in brain injury. In fact, S100B, may play a dual role in the regulation of cell function, being beneficial to cells at low doses, but detrimental when the level increases [99]. Astroglia ability to produce S100B is fundamental to clarify its role after brain injuries. Indeed, it is well known that glial (microglia and astrogli) cells are activated in the white matter (WM) aberrantly after chronic cerebral hypoperfusion [100]. This activation occurs in a manner that predicts the extent and degree of the subsequent WM damage, indicating the significant role played by glial cells population in the pathogenesis of WM lesions. In the vulnerable WM, oligodendroglia’s apoptosis is induced via up-regulation of inflammatory cytokines, such as TNF-α, and free radicals released from activated microglia and astrogli [101]. The concomitant blood–brain barrier disruption [102] allows macromolecules and other blood components - proteases, immunoglobulins, complements, and cytokines - to cross into the perivascular WM tissues where can exert their functions. Furthermore, in a neuronal and astrogli co-culture system, a high concentration of S100 protein up-regulated NO release from the astrogli, which was shown to be neurotoxic [98, 103]. Although the astrogli activation responsible mechanism of low S100 protein concentration is still unclear, it has been postulated a positive feedback loop furtherly stimulating the protein [104, 105]. This exaggerated astrogli activation might lead to a subsequent tissue damage, via cytotoxic cytokines, such as TNFα, and the induction of mediators such as COX2 and iNOS [106, 107]. Indeed, the delayed cerebral damage extension occurred in concomitance with astrogli activation, as well as with increased S100B tissue level in the peri-infarct area. Thus, S100B protein astroglial over-expression is considered to play a pivotal role in infarct expansion via modulation of multiple intracellular signaling pathways and different downstream proteins expression [108, 109]. This hypothesis is supported by data on the protective effect of aruncid acid against astroglial activation and WM lesions during chronic cerebral hypoperfusion, which suppress S100B release and, therefore, prevent an excessive activation potentially harmful for neighboring neurons [108, 110]. Conversely Ellis et al. [111], reported that S100B protein release by rat neonatal neurons, astrocytes, and microglia consequent to an in vitro trauma increases trauma-induced delayed neuronal injury and negates the protective effect of exogenous S100B on neurons.

Finally, the evidences that: i) S100B is not affected by hemolysis; ii) is thermostable for several hours (up to 120-h) at room temperature with no need for immediate analysis and; iii) its short half-life; together suggest S100B measurements as crucial in the emergency and intensive care settings [112, 113]. Based on these characteristics, S100B has been proposed as a promising marker of H-I brain damage providing useful informations about brain lesion extention and highlighting its importance for developing new therapeutic approaches.

CSF was the first among different biological fluids in which S100B role as a marker of active brain damage was shown [114, 115]. In perinatal medicine, CSF protein measurements have been used to monitor infants affected by perinatal asphyxia and post-hemorrhagic ventricular dilatation brain damage. S100B levels correlated with the extent of brain lesions, with long-term prognosis, and with neurological impairment at 1 year of age or death before that time [116-118].

The idea to measure blood S100B, was based on the hypothesis that during active brain injury, at least some of the protein released from the damaged tissue could spread into the systemic circulation [119], as a result also of hemodynamic rearrangement of the blood brain barrier. Indeed, with respect to perinatal medicine, increased blood S100B concentrations were detected 48 to 72 h before any clinical, laboratory, or ultrasound signs of cerebral bleeding (i.e. IVH) in preterm [120], and HIE in full-term infants [121, 122]. In this respect, Nagdyman et al. [122] reported that cord blood S100B concentrations were significantly higher in asphyxiated full-term infants suffering from birth asphyxia and HIE. A longitudinal S100B protein monitoring in peripheral blood from the same authors demonstrated a protein peak concentration 6 h after birth with a S100B progressive decrease at 24 h. The positive predictive value of S100B for HIE with a protein cut-off of 8.5 µg/L at 2 h from birth was 71%, the negative predictive value was 90%, the sensitivity was 71%, and the specificity was 90%. S100B blood concentrations also correlated with abnormal cerebral hemodynamic patterns (increased cerebrovascular resistance) and with the extent of IVH both in preterm and in full-term asphyxiated infants [120, 121]. In asphyxiated full-term infants, an early increase in S100B was found to be predictive of HIE and consequent adverse neurological outcomes [122]. S100B protein has also been used to monitor the occurrence of cerebral complications in CHD infants undergoing extracorporeal membrane oxygenation support to treat respiratory distress [123, 124]. High S100B levels have also been shown in IUGR fetuses with redistribution of fetal-placental blood flow, the so called “brain sparing effect”, correlating with the fetal hemodynamic impairment degree, as indicated by an altered middle cerebral artery Doppler pattern; conversely, in IUGR fetuses without “brain sparing effect” S100B protein showed similar levels to those of non-IUGR fetuses [125]. On this regard, blood S100B was also measured in women whose pregnancies were complicated by IUGR and whose newborns develop IVH [126]. Before clinical, laboratory, and ultrasound patterns are able to detect IVH risk, maternal S100B showed already higher levels in IUGR pregnancies complicated by IVH compared to unaffected pregnancies. At a cut-off of 0.72 µg/L, sensitivity was 100% and specificity was 99.3% for prediction of IVH [126]. S100B levels are also detectable into urine fluid; in particular, in the urine of healthy preterm and term newborns its concentrations correlate with gestational age at sampling, offering a normality reference curve [127]. Birth urine S100B concentrations were significantly higher in preterm newborns that later developed cerebral bleeding and/or brain damage at a stage when all routine clinical, laboratory, and ultrasound investigations were still silent. Urine S100B longitudinal monitoring showed a progressive protein increase with a peak at 72 h from birth. The positive predictive value of S100B for IVH with a protein cut-off of 0.70 µg/L at 2 h from birth was 80.5%, the negative predictive value was 100%, the sensitivity was 100%, and the specificity was 100% [128]. Urine S100B measurement
Activin A on has been also used as an early indicator of neonatal death risk. Moreover, a cross sectional study of urine assessment in 165 preterm newborns, 11 of whom suffered neonatal death within the first week, 121 displayed no overt neurological syndrome, 33 suffered neonatal hypoxia and IVH but not ominous outcome, S100B concentrations were shown to be higher in infants that died within the first week. An S100B concentration cut-off of 12.93 MoM at first urination had a sensitivity of 100% and a specificity of 97.8% for predicting an ominous outcome. The positive predictive value was 78.6%, the negative predictive value was 100% [129]. Urine S100B clinical usefulness for early detection of post-asphyxia brain damage was evaluated in asphyxiated full-term newborns [130]. The concentrations of S100B protein in urine were higher in samples collected in newborns with abnormal neurological findings on follow-up than in samples from those with no sign of disease, or from healthy infants. An S100B concentration cut-off of 0.28 µg/L at first urination had a sensitivity of 100% and a specificity of 87.3% for predicting the development of abnormal neurological findings on follow-up. The sensitivity and specificity of measurements obtained between 12 and 72 hours were up to 100% and 98.2%, respectively [130].

In CHD infants, S100B has been performed with the aim to monitor brain stress at different perioperative phases. Westaby et al., in 1996 [131], in children, reported that S100B blood concentrations increased during CPB when compared to other pre/post-operative phases. Following this first observation, S100B was assessed in CHD infants undergone to open-heart surgery and CPB in the first month of age [132]. Results showed that protein* concentrations, similarly to children, were significantly higher at CPB phase than perioperative phases. Further studies showed that the crucial points of CPB procedure, on S100B, were the length of cooling and rewarming phases [133, 134]. Of note, S100B longitudinal assessment, during open-heart surgery and CPB, was performed in order to monitor positive/side-effects of new CPB therapeutic management of cooling and rewarming phases with vasodilators such as phentolamine [135]. Interestingly, the results on S100B patterns during surgical procedure, suggested abandoning vasodilators administration.

**Activin A**

Activin A is a growth factor composed of two betaA subunits belonging to the transforming growth factor beta (TGF-beta) superfamily of dimeric proteins. Activin A biological activity it has been shown to be mediated by two different types of receptors: the type I (ARI, ARIB) and II receptors (ARI, ARIB), and by two activin-binding proteins, follistatin and follistatin-related gene that inhibit its biological effects. Activin A, its receptors, and its binding proteins are widely distributed throughout the brain [136]. Acute brain injury model studies strongly implicate enhanced activin A expression as a common response to acute neuronal damage of various origins. Hypoxic/ischemic injury, mechanical irritation, and chemical damage of brain evoke a strong up-regulation of activin A [137]. Experimental studies also demonstrated a beneficial role of activin A on neuronal recovery and its robust neuroprotective activities via transduction of different cellular pathways [137, 138]. Activin A measurement, therefore, might represent a potential biochemical indicator of the presence, location, and extent of brain injury thanks to its early induction after brain injury. The diagnosis of subclinical lesions at stages when monitoring procedures are unable to detect brain lesion, might facilitate the establishment of a prognosis by such as approach.

Activin A enhanced expression represents a normal response to acute neuronal damage of various origins [137] however, the functional implications of enhanced activin A expression are not fully understood. In this regard, the current opinion on activin A are supported by: i) its beneficial role to neuronal recovery, ii) its ability to promote neurogenic cell lines and retinal neurons survival, iii) its protective ability on midbrain dopaminergic cultures neurons against neurotoxic damage, iv) its ability to enhance rat embryonic hippocampal neurons in vitro survival, decreasing ischemic brain injury in infant rats, and, v) rescuing striatal neurons against neurotoxic damage in rats [139-144]. In humans, a longitudinal cohort study have collected CSF activin A levels at birth of healthy and asphyxiated full-term newborns newborns affected by HIE, within the first 7-days after birth [143]. The results showed that full-term asphyxiated infants had increased CSF activin-A levels compared to healthy controls, suggesting H-I insult ability to triggers activin A release. Moreover, activin A levels were shown to be higher in asphyxiated infants developing severe HIE compared to the group did not or to controls; elevated CSF activin A levels might be therefore reasonably considered a direct expression of CNS secretion. By the mean of activin A measurement, the early detection of hypoxic ischemic brain lesions was feasible before the presence of related biophysical signs, since newborns with activin A levels above the threshold defined by the receiving operator characteristics curve analysis had a probability of developing HIE as high as 100%, and 0% if levels were unaltered [143]. Activin A blood levels were also found to be significantly higher in preterm newborns developing IVH than in those who did not, and at the cut-off of 0.8 µg/L activin A achieved a sensitivity of 100% and a specificity of 93% as a single marker for the prediction of IVH in preterm newborns [144]. More recently, in asphyxiated full-term newborns activin A urine levels have shown to be an available diagnostic tool for HIE prediction. Activin A urine levels were, in fact, significantly (P<0.0001) higher in asphyxiated newborns with moderate or severe HIE than in those with absent or mild HIE, and controls. Activin A concentration of >0.08 ng/L at first void had a sensitivity of 83.3 % and a specificity of 100 % for predicting moderate or severe HIE [145].

In CHD infants, underwent open-heart surgery with CPB, activin A levels was assessed in 45 infants (age <1 year), 9 of whom experienced neurologic injury, on day 7 after the surgical procedure [146]. Blood samples were drawn at 5 predetermined time-points (before surgery, during surgery before CPB, at the end of CPB, at the end of surgery, and at 12 h after surgery). Data obtained have shown that activin A levels significantly increased during surgery till a maximum at the end of CPB. In addition, infants developing abnormal neurologic sequelae had activin A levels significantly higher than patients with normal neurologic outcome at all evaluated times, but not before surgery. Activin A at a cut-
off of 0.94 µg/L had a sensitivity of 100% and a specificity of 100% as a single marker for predicting neurologic abnormalities. These findings supported the notion that: i) activin A increases in children who experience poor neurologic outcomes following open-heart surgery and; ii) its assay may help in early identification of infants at risk for brain damage [144].

**Adrenomedullin**

Adrenomedullin is a novel hypotensive peptide isolated from human pheochromocytoma Kitamura et al. in 1993 [147]. Intensive investigation in the ensuing years, towards its implication in the regulation of cerebrovascular, cardiovascular and pressure/volume homeostasis, and its potential role in the pathophysiology of heart diseases, has been performed. In human, AM peptide has been shown to be composed of 52 aminoacids, which sequence is highly conserved across species, particularly within its six-residue ring and C-terminal amide structures, essential for AM bioactivity. AM has been detected in a wide variety of tissues such as adrenal and pituitary glands, cardiac atrium and ventricle, lung, kidney and vasculature [148]. AM gene expression has been found in vascular tissue, ventricle, kidney and lung, suggesting these tissues as potential region of secretion [149].

Among different AM functions, to date still matter of investigation, the main proven is vasodilatation [147], although neuromodulation [150] and inhibition of apoptosis [151, 152] have been reported. AM gene expression has been shown to be up-regulated by hypoxia [153, 154] and inflammation [155], both of which shown the ability to activate the neo-vascularization process. To this regard, AM gene knockout mice data have demonstrated a role for AM in the formation of vasculature in embryos [156-158]. AM was first identified in the hypothalamus [159] and only following studies reveals its presence into the whole CNS [160]. The caudate-putamen was the only cerebral area where AM was found in the neuronal nuclei [159], which also correspond to the hypoxia most sensitive brain region [161]. AM is, in fact involved in hypoxia response, at least in part by the transcription of the Hypoxia-Inducible Factor-1, which enhances AM expression and stabilizes AM mRNA [161]. Moreover, it has been reported that H-I up-regulates AM expression in the cerebral cortex [162] and in the caudate-putamen [162] of the adult rat brain. In neonatal rat brain, genomic response to hypoxia corroborated the aforementioned findings, because AM gene was found to be 8-9 folds up-regulated by hypoxia via HIF-1 in neuron cells [163]. AM is mainly expressed in neurons and endothelium in CNS [160], and it has been reported that transient ischemia boosted AM expression for more than 15 days [153]. In addition, evidences that exogenous AM administration after 20min reduced the infarct area in mice, and promoted vascular regeneration and functional recovery indicating a neuro-protective and vasculo-neuro-regenerative roles of AM [164-166].

In fetus, AM putative role has been suggested in fetal cardiovascular adaptation and in placental perfusion, because of higher AM concentration observed in IUGR infants, both in cord and peripheral blood, compared to unaffected newborns [167-170]. In addition, AM was assessed from birth within the first 12-hours, in full-term asphyxiated newborns with IVH, showing significantly higher AM levels in asphyxiated infants compared to those without IVH, or healthy controls. AM blood concentrations were found to be increased in HIE infants according to the severity of H-I insults [171, 172]. In preterm infants, blood AM concentrations measured within the first 6 hours from birth, have been found to be significantly higher in newborns who later developed IVH than in the rest of patients [173]. On the light of these findings, it has been suggested that AM might have a role in the loss of cerebral vascular autoregulation in response to hypoxia, and be therefore a useful marker able to depict neonates, among those at risk, presenting adverse neurological outcomes [38-42].

In addition, a tiny increment in AM plasma levels, it has been reported between the coronary sinus and aorta in heart failure [174], suggesting a putative cardiac involvement in the increase levels observed in such disease. In according with this finding is AM immunoreactivity and gene expression increase in the cardiac ventricles of animals and patients with heart failure [174-178]. In a heart failure rat model, it has been shown that the main source cardiac AM might be myocardial blood vessels [174-178].

Finally, Florio et al. [178], in CHD 50 infants (age <1 year), investigated whether CPB affects cerebrovascular resistance (Doppler velocimetry waveform patterns in middle cerebral artery, MCA PI) and AM plasma levels in samples collected at five predetermined timepoints (before surgery, during surgery before CPB, at the end of CPB, at the end of surgery, and at 12 h after surgery). Primary endpoint was the occurrence of brain injury at 2-year follow-up. In this setting, forty infants showed no overt neurological injury, while 10 were affected by brain damage. Results reported the highest MCA PI values and lowest AM levels, at the end of CPB and of the surgical procedure, in brain damaged patients. As single markers able to predict neurological abnormalities, AM achieved a sensitivity of 100% and a specificity of 73.0%, whilst MCA PI reached a sensitivity of 100% and a specificity of 56.8% [178].

**CONCLUSION**

CNS monitoring and brain damage early detection/prevention, in infants non-CHD or CHD complicated by PA, still remain a challenging task for experts. In this regard, the multidisciplinary approach (i.e. laboratory, intensive care medicine, pediatric, neuroscience, heart surgery, cardiology etc.) allowed obtaining relevant results. This holds for the introduction in daily practice of early selective or total body cooling in the treatment of non-CHD full term infants complicated by PA. Although cooling exerted an important role in reducing the incidence of neurological sequelae in HIE infants, these encouraging results have to be confirmed on the severe HIE forms in which are to date lacking. Furthermore, the choice of which infant has to be cooled or not is an issue that deserves further investigations. Identically, in CHD infants the possibility to detect, at the due time, the cases at risk of interventional complications such as low congestive heart failure and H-I insult are fairly remote. In this setting, from a clinical point of view the use of biochemical markers, constituents of nervous system, might be useful for early detection of: i) PA
candidates suitable for cooling procedure, ii) potential side-effects of risky therapeutic strategies (i.e. cooling, open heart surgery and CPB) on CNS, and iii) cases at risk for perioperative complications.

However, from a laboratory point of view, biochemical markers have to fulfill the following items, strategical for their validation in clinical practice: i) assessment method with a good reproducibility, sensitivity and specificity; ii) the possibility of detection in a variety of biological fluids in order to reduce in non-CHD newborns stress due to sampling modalities; iii) possibility of longitudinal monitoring, relevant both for non-CHD and CHD patients; iv) low costs, and v) well-established and validated use as an early and quantitative marker of brain lesions/damage. All together these items constitute the – so called – “optimality concept” for biochemical markers inclusion in evidence-based guidelines. Currently, our conclusion, suggest that only few have been assessed under all these points of view and no-one is able to reach the optimality concept. Bearing in mind that we are not claiming for one specific marker of being of major clinical significance, however, there are some laboratory issues that need further elucidation. This holds for the possibility of biomarker assessment in different biological fluids and the interval from sampling to obtain result. These points are fulfilled by S100B protein because of its assessment in urine and saliva fluids, whilst, to date activin A and AM have been measured in urine fluid but not in saliva. Furthermore, while activin A and AM results can be obtained within 6 hours (ELISA method), S100B results can be obtained within 2 hours (LIAISON method): this latter point might be crucial for early detection of the cases to treat.

Another relevant point such as cost/benefit of each biochemical marker is lower when compared with any standard monitoring procedure currently used for brain monitoring in sick infants and children.

In conclusion, biochemical markers monitoring in non-CHD and CHD infants can represent, together with standard monitoring procedures, a winner issue in the early detection of CNS injury following PA. Further multicenter investigations will elucidate the role of these biomarkers in clinical guidelines.

**LIST OF ABBREVIATIONS**

AM = Adrenomedullin  
CHD = Congenital Heart Disease  
CNS = Central Nervous System  
CPB = Cardiopulmonary Bypass  
CSF = Cerebrospinal Fluid  
DHCA = Deep hypothermia Circulatory Arrest  
H-I = Hypoxic-Ischemic  
HIE = Hypoxic Ischemic Encephalopathy  
NO = Nitric Oxide  
PA = Perinatal Asphyxia  
PVL = Periventricular Leukomalacia  
WM = White Matter

**CONFLICT OF INTEREST**

The authors confirm that this article content has no conflict of interest.

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