[Frontiers in Bioscience S2, 47-72, January 1, 2010]

Biochemical markers of perinatal brain damage

Pasquale Florio[1], Raul Abella[2], Emanuela Marinoni[3], Romolo Di Iorio[3], Giovanni Li Volti[4], Fabio Galvano[4], Giacomo Pongiglione[5], Alessandro Frigiola[2], Serena Pinzauti[1], Felice Petraglia[1], Diego Gazzolo[5,6]

[1]Department of Pediatrics, Obstetrics and Reproductive Medicine, University of Siena, Siena, Italy, [2]Department of Cardiac Surgery S. Donato Milanese University Hospital, S. Donato Milanese, Italy, [3]Laboratory of Perinatal Medicine and Molecular Biology, and Department of Internal Medicine University "La Sapienza" Rome, Italy, [4]Department of Biological Chemistry, Medicinal Chemistry and Molecular Biology, University of Catania, Italy, [5]Department of Pediatrics, Neuroscience and Cardiovascular Surgery, G. Gaslini Children's Hospital University of Genoa, Genoa – Italy, [6]Department of Fetal, Maternal and Neonatal Health, G. Garibaldi Hospital Catania , Italy

TABLE OF CONTENTS

1. ABSTRACT

Hypoxia-ischemia constitutes a risk in infants by altering cerebral blood flow regulatory mechanisms and causing loss of cerebral vascular auto-regulation. Hypotension, cerebral ischemia, and reperfusion are the main events involved in vascular auto-regulation leading to cell death and tissue damage. These dramatic phenomena represent a common repertoire in infants complicated by perinatal acute or chronic hypoxia. To date, despite accurate perinatal and intra-operative monitoring, the post-insult period is crucial, since clinical symptoms and monitoring parameters may be of no avail and therapeutic window for pharmacological intervention (6-12 hours) may be limited, at a time when brain damage is already occurring. Therefore, the measurement of circulating biochemical markers of brain damage, such as vasoactive agents and nervous tissue peptides is eagerly awaited in clinical practice to detect high risk infants. The present review is aimed at investigating the role as circulating biochemical markers such as adrenomedullin, S100B, activin A, neuronal specific enolase (NSE), glial fibrillary acid protein (GFAP), in the cascade of events leading to ischemia reperfusion injury in infants complicated by perinatal asphyxia.

2. INTRODUCTION

One of the main clinical problems related to hypoxic-ischemic (H-I) damage is the fact that such a disease is associated with delayed motor development and neurological abnormalities (1). Therefore, the early monitoring procedure needs to be as accurate as possible, since it is at this crucial time, when clinical symptoms and monitoring parameters are hidden by sedation and therapeutic strategies effects, that brain damage may occur or be at sub-clinical stage.

The measurement of quantitative parameters, i.e. biochemical markers of brain damage, to diagnose sub-clinical lesions at stages when monitoring procedures are still unable to detect brain lesion could be especially useful in the brain injury prevention and/or management. This possibility relies on the growing number of evidences reporting that the brain produces several locally expressed factors following brain injury, and that they may be released into bloodstream with the opportunity to be assayed (2,3). Indeed, several evidences have also suggested to use the measurement of those brain factors as biochemical indexes: 1) for detecting cases at risk of adverse neurological outcome, 2) to know the timing of insults damaging central nervous system (CNS) and, 3) to diagnose sub-clinical lesions at stages when monitoring procedures are still unable to detect brain lesion, as early as possible, with respect to future measures of prevention (2).

The present review is aimed at investigating the role as circulating biochemical markers such as adrenomedullin (AM), a vasoactive peptide, S100B, a calcium binding protein, activin A, NSE, GFAP in the cascade of events leading to ischemia reperfusion injury in infants complicated by perinatal asphyxia. An overview on epidemiological data, pathophysiology and clinical and experimental findings is also offered.

3. EPIDEMIOLOGY OF PERINATAL ASPHYXIA

Epidemiological studies conducted in the last decades have shown that, despite technological improvement in high-risk newborns and children management, the pattern of neurological handicap incidence in developed countries has a flat trend (4).

Perinatal asphyxia occurs in 0.2-0.4% of full-term births, of these 20% suffer mortal hypoxic-ischemic encephalopathy (HIE), of the survivors, 25% exhibit permanent neuropsychological deficits (5). Evaluation of peripartum factors for postnatal handicaps has to be subdivided in two major chapters: preterm delivery and feto-neonatal H-I injury during labor and delivery in term fetuses. It should be pointed out that handicaps occurring in postnatal period are mainly due to prenatal events such as intrauterine growth retardation (IUGR). When IUGR and prematurity are associated, neurological handicaps in survivors can increase up to 30-45%. In fact, very low birth weight infants (less than 1500 g), although represent 0.2-1.2% of neonatal population, account for 2/3 of neonatal mortality and morbidity (6). About 10% of infants, in the developed countries, show an IUGR. Fifteen-twenty percent of perinatal asphyxia complications in term and preterm newborns are represented by CNS diseases with high morbidity, such as HIE and intraventricular hemorrhage (IVH). Nonetheless, it has been stated that the majority of neurological abnormalities, detected during childhood, are already there in the first weeks after birth. Knowledge on the timing of adverse insults is, therefore, of utmost importance with respect to future measures of prevention although such a knowledge is, at this stage, still incomplete and matter of debate. There is clinical evidence suggesting that fetal pre-exposure to adverse intra-uterine conditions plays a causal role in antenatal CNS injury and perinatal mortality (7) as well as the reported contribution of asphyxia at birth to cerebral palsy in infants born at term varies from 8% to 28% (5,8,9).

Preterm birth accounts for most cases of perinatal mortality and for 40% of neurologically handicapped children. According to population studies, by Hagberg B. *et al* (10), 60% of neurological handicaps in preterm infants are attributable to peri-neonatal events, 10% are of antenatal origin, and 30% are of generally unknown origin. In infants born at term, 50% of cases of cerebral palsy have a prenatal etiology, 36% are of peri-/neonatal origin, and 14% of cases are of unknown etiology (10).

4. PATHOPHYSIOLOGY OF BRAIN DAMAGE FOLLOWING H-I INSULT

The developing fetal brain is highly dependent on sustained blood flow, due to the lack of its own energy and nutrient reserves. During H-I insult, several cellular mechanisms are set in motion that trigger cell damage (11). The severity, intensity and timing of asphyxia, as well as selective ischemic vulnerability and the immaturity of the brain, determines the extension and degree of severity of the ensuing damage (12-14). There are many circumstances which render the developing brain especially vulnerable to ischemia (1,15-17). A close relationship between H-I insult and the development of brain damage has also been demonstrated (18,19). Thus, in the preterm infant, peri-intraventricular hemorrhage and periventricular leukomalacia are the most characteristic brain lesions, secondary to perinatal H-I insult. On the other hand, 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 injuries.

Etiological pathways for brain damage in preterm newborns are complex and difficult to be explored. It is likely that different patterns of risk factors may act in different “susceptibility windows of pregnancy”. The resulting brain damage in the newborn could represent the final outcome of exposure to several combinations of risk factors in the same pathway or in different pathways, and can change according to the gestational age. Increasing the knowledge on the pathogenesis of brain damage is essential in order to implement effective prevention strategies. The ischemic theory focuses on the role of H-I resulting from perinatal complication, often present in preterm newborns,

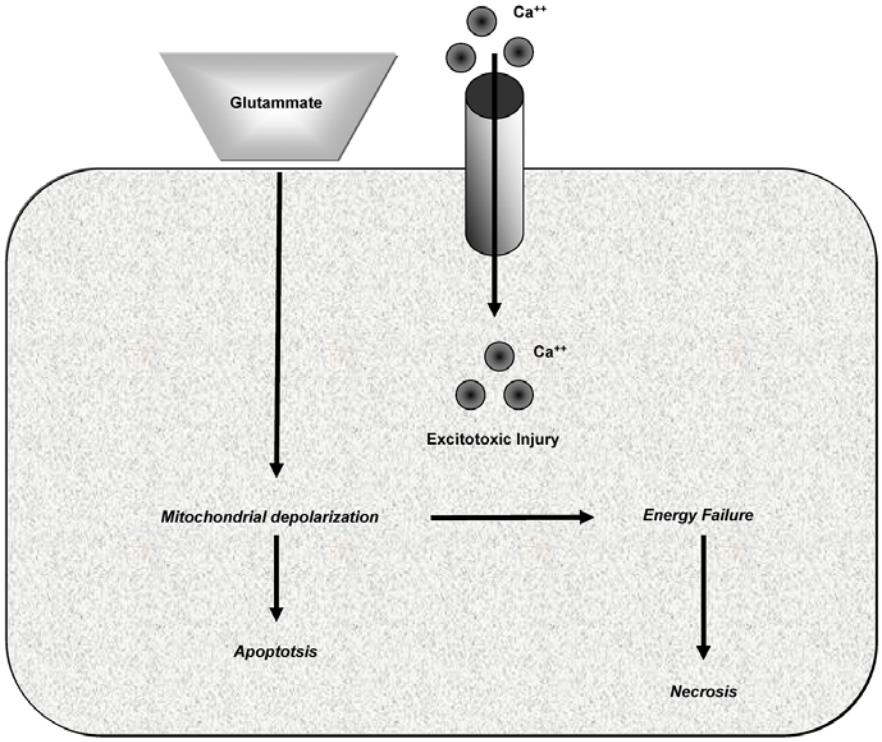

Figure 1. Schematic representation of the major pathway involved in the pathophysiology of brain injury.

as a determinant of breakdown of neuronal metabolism and the subsequent cerebral damage (18,20,21). Evidence supporting this model stems from several studies which demonstrated that: a) a severe and acute loss of oxygen in cerebral tissue leads to a reduced protein synthesis and neuronal death within minutes from the insult (22) b) anoxia also acts as a triggering factor for an uncontrolled and elevated release of excitatory neurotransmitters, which contribute to brain damage even with long-time kinesis (23) c) apoptosis could influence the result of ischemia when this is not sufficiently severe to determine a tissue necrosis (24). These evidences are based on the results of several experiments using animal models: the reduction of glucose supply and the lower availability of ATP in neuronal cells produce a Ca^{++} overload in the citoplasmatic fluids that activates several lythic enzymes and, at the same time, reduce the production of antioxidant molecules and structural proteins which are useful for cellular homeostasis (25-27). One of the consequences of the "calcium overflow", provoked by anoxia in affected cells, is represented by a release of excitatory neurotransmitters which, in turn, hyperstimulate postsynaptic neurons and oligodendroglia through the opening of specific receptors, so allowing a further entrance of calcium within these cells (28). Cell damage is enhanced by the production of free radicals and nitric oxide that attack the structural components of the neurons (29,30). Free radicals, together with other toxic factors such as histamine and serotonin, could be produced by the activation of mast cells in the brain. The role of these cells in brain damage has been supported by the observation that IL-9, a cytokine that binds to the receptor of these cells, exacerbates the excitotoxic damage induced by ibotenate in mice (31). Apoptosis as a result of ischemia has been hypothesized on the basis of the apoptotic bodies found in the brain of asphyxiated animals. Apoptosis is thought to be provoked by mild or moderate ischemia sufficient to determine the lesion of vital components, such as mitochondria, that release pro-apoptotic molecules (i.e. cytochrome c) in the cytosol (32). Although these observations demonstrate that H-I can produce brain damage, they do not completely explain why asphyxiated preterm newborns present a site specificity of damage, slightly different from that of term infants and adults suffering of stroke following an acute H-I event (33-37).

4.1. Pathogenesis of Hypoxic–Ischemic Cerebral Injury

The principal pathogenetic mechanism underlying most of the neuropathology attributed to intrapartum H-I is impaired cerebral blood flow (CBF). This is most likely to occur as a consequence of interruption in placental blood flow and gas exchange, often referred to as "asphyxia" or severe fetal acidemia. The latter is defined as a fetal umbilical arterial pH less than 7.00 (38). At the cellular level, the reduction in cerebral blood flow and oxygen delivery initiates a cascade of deleterious biochemical events. Depletion of oxygen precludes oxidative phosphorylation and results in a switch to anaerobic metabolism. This is an energy inefficient state resulting in rapid depletion of high-energy phosphate reserves including ATP, accumulation of lactic acid and the inability to maintain cellular functions (39). Transcellular ion pump failure results in the intracellular accumulation of Na^{+}, Ca^{++} and water (cytotoxic edema). The membrane

depolarization results in a release of excitatory neurotransmitters and specifically glutamate from axon terminals. The glutamate then activates specific cell surface receptors resulting in an influx of Ca^{++} (Figure 1). As a consequence of increased Ca^{++} influx, there is the triggering of a complex biochemical cascade which includes the production of S100B leading to the activation of the nitric oxide (NO) signaling pathway via the p38MAPK, JNK and ROS dependent mechanisms (40,41). Concomitantly to the activation of NO pathway, there is a significant increase of activin A mediated by increased radicals formation which leads to a further increase of Ca^{++} which continues to perpetrate the above mentioned mechanisms (42). In addition, intracellular Ca^{++} induces the production of NO, a free radical that diffuses to adjacent cells susceptible to NO toxicity. The combined effects of cellular energy failure, acidosis, glutamate release, intracellular Ca^{++} accumulation, lipid peroxidation and NO neurotoxicity serve to disrupt essential components of the cell with its ultimate death (18) which results in a significant increase of specific CNS biochemical markers such as NSE and GFAP (43,44). On the other hand, AM is released from the extracellular space, in the attempt to attenuate Ca^{++} release into the cytosolic compartment and the activation of caspases activation which in turn leads to apoptosis (45).

4.2. Delayed (secondary) Brain Damage

Following resuscitation, which may occur *in utero* or postnatally in the delivery room, cerebral oxygenation and perfusion is restored. During this recovery phase, the concentrations of phosphorus metabolites and the intracellular pH return to baseline. However, the process of cerebral energy failure recurs from 6 to 48 h later in a second phase of injury. This phase is characterized by a decrease in the ratio of phosphocreatine/inorganic phosphate, with an unchanged intracellular pH, stable cardiorespiratory status and contributes to further brain injury (15,39). In the human infant, the severity of the second energy failure is correlated with adverse neurodevelopmental outcome at 1 and 4 years (46). The mechanisms of secondary energy failure may involve mitochondrial dysfunction secondary to extended reactions from primary insults (e.g., calcium influx, excitatory neurotoxicity, oxygen free radicals or NO formation). Indeed, mitochondria play a key role in determining the fate of neurons following H-I. Thus, translocation of apoptotic triggering proteins such as cytochrome c from the mitochondria to the cytoplasm can activate a cascade of proteolytic enzymes termed caspases or cysteine proteases that eventually trigger nuclear fragmentation (44). Recent evidence also suggests that circulatory and endogenous inflammatory cells/mediators also contribute to ongoing brain injury (47). To discuss this process further, some basic mechanisms of ongoing injury are outlined next.

4.3. Excitatory Neurotransmitter Release

Excitotoxicity as a mechanism for neuronal injury after H-I insult has received a great deal of attention during the last decade, and much has been elucidated regarding this process in the immature brain. Most of what we know comes from animal studies (mainly rodent) but studies in the human neonate have provided some corroboration.

Glutamate is a major excitatory amino acid within brain. The action of glutamate is mediated by a number of receptor subtypes with the NMDA receptor predominating in developing brain and is increased in areas of active development, e.g., striatum or hippocampus. During H-I, glutamate accumulates within the synaptic cleft secondary to increased release from axon terminals as well as impaired reuptake at presynaptic nerve endings. When glutamate is released from presynaptic vesicles into the synapse, it can stimulate postsynaptic receptors (NMDA, AMPA, or kainate). Removal of glutamate from the synapse is dependent on glutamate transporters present mainly on glial cells. The glia convert glutamate to glutamine, glutamine is transported out of the glia and into neurons, and the neurons convert glutamine back to glutamate (48) This process requires intact cellular energy machinery and function, and can be disrupted by any process that causes energy failure (49-51) including glucose deprivation or H-I. (52) Since the fetus is adapted to a low oxygen tension and has a low cerebral basal energy consumption compared with the mature organism, this could explain the more delayed energy failure that occurs in immature brain.

When the neurotransmitter recognition site is activated by glutamate, the ion channel allows influx of calcium and sodium. The increase in intracellular calcium that results is the stimulus for a multitude of downstream events, including regulation of transcription factors, cell cycle regulation, and DNA replication. (53) The NMDA receptor is relatively over-expressed in the developing brain compared with the adult brain (54).

The higher density and activity of glutamate receptors in the perinatal period creates a potential for a more devastating effect when energy failure does occur. The same NMDA receptor activation which allows for plasticity and synaptogenesis can, in the setting of H-I, lead to massive Na^{+} and water influx, cellular swelling and necrosis, pathologically elevated intracellular calcium and its associated mitochondrial dysfunction, energy failure, and apoptosis. As neurons die, degradative enzymes are released, leading to a "spiral of death" (49) potentiated further by inhibition or even reversal of glutamate reuptake by glia. Since glycolytic metabolism provides the ATP that powers the glial glutamate transporter, (55) deprivation of glucose (which occurs in ischemia) will lead to high synaptic glutamate levels. H-I in rat hippocampal neurons leads to marked reduction in the activity of the pumps which remove glutamate from the synapse, (56) and the presynaptic glial glutamate transporter in immature rats subjected to unilateral carotid artery ligation and hypoxia is severely affected (57).

4.4. Formation of Free Radicals

The concepts of excitotoxicity and oxidative stress are inextricably linked, and many of the nuances of this complex relationship are still being clarified. Oxidative stress is a general term for the increase in free radical

production as a result of oxidative metabolism under pathologic conditions. The concept of ischemia/reperfusion whereby glucose and oxygen deprivation lead to primary cell death and reperfusion and re-oxygenation lead to secondary cell loss (7) is fundamental to the understanding of oxidative stress. In aerobic cells, oxygen free radicals (i.e., O_2^-, H_2O_2, OH^-) are produced within the cytoplasm and mitochondria. Under physiologic condition, oxygen free radicals are destroyed rapidly by endogenous antioxidants (i.e., superoxide dismutase, endoperoxidase, catalase) and scavengers (i.e., cholesterol, alpha-tocopherol, ascorbic acid, glutathione) (15,44,46,47). When oxygen floods the microenvironment of cells that have been damaged by hypoxia, mitochondrial oxidative phosphorylation is overwhelmed and reactive oxygen species accumulate (58). Antioxidant defenses are depleted and free radicals damage the cell by peroxidation of lipid membranes, alteration of membrane potentials, activation of pro-apoptotic mediators, and direct DNA and protein damage. Excitotoxicity causes energy depletion, mitochondrial dysfunction leading to the generation of free radicals such as superoxide, nitric oxide derivatives, and the highly reactive hydroxyl radical. Free radicals in turn alter membrane and pump function, allowing for more glutamate release and NMDA receptor activation and leading to more excitotoxicity. Because of its high lipid (specifically, polyunsaturated fatty acid) content, the brain is particularly susceptible to free radical attack and lipid peroxidation (59). This heightened vulnerability is magnified in the term newborn brain for several reasons. First, the polyunsaturated fatty acid content of the brain increases during gestation (60). There is a basal level of lipid peroxidation under normal conditions that is higher in term than preterm brain (59). Lipid peroxidation leads to the activation of phospholipases that increase free radical production. Under hypoxic conditions, free radical accumulation in brain occurs, (61) and hypoxic tissue undergoes peroxidation much faster than normoxic tissue (59). Second, the immature brain has immature antioxidant defenses. Specifically, the antioxidant enzyme systems superoxide dismutase, catalase, and glutathione peroxidase display less activity in the immature than the mature rat brain (62) Third, the newborn brain is rich in free iron relative to the adult brain (63). Developmentally this is advantageous because iron is a cofactor in many enzymatic reactions that correspond to neuronal growth and differentiation. However, free iron can catalyze the production of various reactive oxygen species. Increased free iron is detectable in the plasma (64) and CSF of asphyxiated newborns (65). In the rat, brain regions with high iron content are more vulnerable to injury (66) and desferoxamine, an iron chelator, is protective against H-I in animal models (67,68).

The damaging potential of abundant iron and immaturity of the enzymatic oxidant defenses of the immature brain are tightly interrelated. Copper-zinc superoxide dismutase (SOD-1) is the cytosolic enzyme responsible for conversion of superoxide to hydrogen peroxide. Hydrogen peroxide is further reduced to water by glutathione peroxidase or catalase, or alternatively it can be converted to the hydroxyl radical in the presence of ferrous iron. Enhanced glutathione peroxidase is protective when immature neurons *in vitro* are exposed to hydrogen peroxide (69). Therefore, an imbalance of enzymatic maturity can be invoked to explain the maturational differences, lack of sufficient glutathione peroxidase activity in the immature brain can lead to accumulation of hydrogen peroxide (70) and lipid peroxidation products. Both free radical scavengers (PBN, a nitrone spin-trap that converts free radicals to stable adducts) and metal chelators (desferoxamine and TPEN) have been shown to protect neurons from injury mediated by hydrogen peroxide *in vitro* (67,71) and *in vivo* (72). These interventions also protected neurons from NMDA-induced toxicity, strengthening the link between excitotoxicity and oxidative stress (67). The link between excitotoxicity and free radical injury is well exemplified in studies of the role of NO, another reactive oxygen species, in H-I brain injury. NO can function both physiologically and pathologically. Produced constitutively in endothelial cells, astrocytes, and neurons in response to an increase in intracellular calcium, it has a role in pulmonary, systemic, and cerebral vasodilation, and is thought to exert a compensatory vascular effect after ischemia during reperfusion. iNOS is produced in macrophages, endothelial cells, neurons, and astrocytes in response to stress. Hypoxia induces generation of NO in the cortex of newborn guinea pigs, and NO can modify the glycine-binding site of the NMDA receptor of cortical neurons during hypoxia, facilitating calcium entry and enhancing excitotoxicity (73). Neurons that express neuronal NOS (nNOS) in the striatum are selectively resistant to H-I injury (74). Neuronal NOS expression corresponds anatomically to immature NMDA receptor expression, especially in the basal ganglia (75) and disruption of the nNOS gene (74) and pharmacologic inhibition of nNOS (76) both ameliorate H-I injury. In addition to their participation in oxidative injury and in the excitotoxic cascade, NO and NOS have been implicated in the programmed cell death that results from H-I injury. Inhibition of nNOS in newborn piglets prevents the increase in caspase-3 (the so-called “death effector”) activity and subsequent DNA fragmentation (77,78). In a separate study of hypoxia in newborn piglets NOS inhibition can block activation of ERK and JNK, (79) two of the mitogen-activated protein kinase (MAPK) family that mediates signal transduction from cell surface to nucleus and thereby regulates programmed cell death (80,81).

4.5. Inflammatory Mediators

Inflammatory cytokines may have a direct toxic effect via increased production of inducible nitric oxide synthase, cyclooxygenase and free radical release, or indirectly via induction of glial cells to produce neurotoxic factors such as excitatory amino acids. It has been proposed that cytokines may be the final common mediators of brain injury that is initiated by H-I, reperfusion, and infection (82). Cytokines are polypeptides that act either systemically or in a local fashion to guide the cellular response to inflammation, H-I, infection, and a variety of other stressors. Their cellular targets are myriad and located throughout the body, including astrocytes, neurons, microglia, and endothelium of the CNS. The cytokines IL-

1beta, TNF-alpha, IL-6, and IL-8 have been implicated clinically in the pathologic effects of brain inflammation (83,84). In addition, mediators such as platelet activating factor (PAF), arachidonic acid, and their metabolites (prostaglandins, leukotrienes, thromboxanes, cyclo-oxygenase) are involved in the inflammatory response during the evolution of brain injury after ischemia/reperfusion. The cellular origin of inflammatory mediators that appear to exacerbate H-I brain injury is still unclear. There is a role for mediators that are produced systemically (by the mother or by the fetus itself) and affect the CNS either through vascular mechanisms or by entry across the brain blood barrier and direct action on brain parenchyma. However, microglia, the resident macrophages of the CNS, are activated by H-I and can release glutamate, free radicals, and nitric oxide (85). In fact, microglia are activated experimentally by ibotenate, an exogeneous excitotoxin (86). Drugs that block resident microglial and blood-derived monocyte activation (such as minocycline or chloroquine) protect the newborn brain from this excitotoxin (87).

The wide variability in the effect of H-I on the newborn brain highlights the probability that genetic factors play a significant role. Rodent models of H-I show wide interstrain variability in the severity of injury after an identical insult (88,89). Possibilities have emerged regarding genetic predisposition to brain injury, (90) and once again the perinatal period is of particular interest and represents one of the most likely time periods for a genetic modifier to present itself. For instance, genetic abnormalities causing a hypercoagulable tendency seem to increase risk for stroke in adults only if they are present in combination (91). However, the same mutation in a single gene in the neonate can be associated with increased risk for ischemic stroke (92). Gender differences with respect to the response to H-I have also been observed (93). It is reasonable to assume that there are a variety of genetic factors that may achieve their highest potential to manifest during the perinatal period. Taken as a whole, the initiation and evolution of brain injury in the term infant after a H-I insult is a vastly complex process, with contributing mechanisms densely interwoven to create a picture in which it is challenging to find a common thread. Recent investigations have focused on how the different yet related processes of excitotoxicity, oxidative stress, and inflammation come together in the neonate to produce a picture that is unique from that of the adult. It is by continuing to focus on this synthesis that we will arrive at an understanding of how neonatal encephalopathy occurs, and what we can do to prevent it. Thus, inflammatory cytokines appear to exert both beneficial and deleterious effects following ischemia. This dual effect will likely complicate the task of developing targeted interventions against the inflammatory response.

5. ROLE OF NEAR-INFRARED SPECTROMETRY (NIRS) TO EVALUATE CEREBRAL OXYGENATION

A basic aim of neonatal intensive care is the preservation of an adequate oxygen contribute to the tissues, particularly the brain (94). This process depends on sufficient blood oxygen content, blood flow to the tissues and the ability of cells to extract and utilise oxygen, the first depends on ventilation and haemoglobin concentration and type, while blood flow depends on cardiac output. Alteration of this fine equilibrium can led to a brain damage following H-I insult.

Although improvements in perinatal care have been carried on, H-I injury to the brain remains an important cause of death and permanent neurodevelopmental impairment in both term and preterm infants. As the first hours after insult are fundamental to perform neuroprotective strategies, this condition forces to an early discovery and treatment of infants at risk.

At the moment, measurements of systemic variables, as arterial oxygen saturation (SaO2), by pulse-oximetry, heart rate and blood pressure, by electrocardiography (ECG) and invasive and non-invasive blood pressure techniques, offer indications of the cerebral hemodynamic reaction to systemic hemodynamic events. Other standard imaging techniques, like cranial ultrasound and MRI (95-97), neurophysiology evoked potentials (98) and electroencephalography (EEG) (99-100), appear more usefull for prognosis than diagnosis and are not always available in the Neonatal Intensive Care Units (NICUs). Despite these assessing methods cerebral damage recurrently occurs even in infants whose systemic parameters were stable and, since these conventional procedures have failed to provide effective strategies for the prevention of brain injury, new methods for assessing the adequacy of the cerebral circulation of the neonate have been investigated. On this regard, Near-Infrared Spectroscopy (NIRS) represents a developmental and emerging technique that can be proposed as a potential prognostic tool for brain monitoring. It was firstly introduced in 1977 (101), even if it found the first medical application only in 1985 to study human brain hemodynamic (102) and it has been widely improved during the following years. NIRS allows non-invasive and real-time monitoring of brain oxygenation in newborns complicated by acute and chronic hypoxia, without an additional stress for infants. The technique is based on the relative transparency of tissue to light in the near infrared spectrum (700-1000 nm) and on the presence of fixed or mobile chromophores localized in the tissue, as cytochrome aa_3 or haemoglobin (Hb) respectively, which are capable to NIR light absorption and to transmit light at quantifiably different amounts depending on their oxygenation state. However, the changes in cerebral cytochrome in infants was found inconsistent and insignificant and at the moment only Hb is used as indicator of cerebral oxygenation. NIRS allows directly measuring changes in oxyhaemogloin (ΔHbO2) and deoxyhaemoglobin (ΔHb) and evaluating the absolute value of regional tissue oxygen saturation (rSO2), it also provides other parameters, as tissue oxygenation index (TOI), cerebral blood flow (CBF), cerebral blood volume (CBV) and cerebral venous oxygen saturation (SvO2), which can be derived from the previous data. Among that information, rSO2 and SvO2 measurements have been demonstrated as the fundamental values that are provided by NIRS compared with other techniques. Indeed,

a comparison between NIRS and pulse-oxymetry demonstrated a strong correlation between rSO2 and SaO2 for large changes in oxygenation, but poor correlation for smaller changes, as SaO2 represents a whole-body parameter that does not necessarily reflect the regional oxygenation status (103). In fact, if hypoxia occurs SaO2 may decrease, reflecting the systemic circulation of the human body, but if a system circulation disturbance does not happen, also in presence of a cerebral ischemia, SaO2 will not decrease. At the same, the combined estimation of SaO2 and SvO2 is essential to evaluate the adequacy of O2 offer compared with O2 supply of the tissue, as SaO2 alone provides only a compute of the O2 offered to the tissue without information about the equilibrium between offer and supply.

Recent studies on newborn population focused on NIRS measurements to establish possible differences among physiologic and pathologic conditions. In both condition spontaneous oscillations of the concentrations of HbO2 and Hb have been demonstrated and they probably reflect a spontaneous vasomotion (104). Besides, it has been demonstrated that in healthy term infants rSO2 rapidly increase over the 15 minutes after birth, reaching a value of 64±8%, without significant variations in the following hours (105). Several studies on pathological infant (preterm neonates and hypoxic-ischemic new-borns) population have been carried on. NIRS values were significantly higher in preterm than tern neonates, according to other studies that reported continue changes in rSO2 values during the first three days after birth, as result of increase in cerebral blood flow during that period (106). The fact that in healthy infants, cerebral oxygenation values differed in preterm and term newborns mainly between 32 and 36 weeks is consistent with the notion that in late-preterm the central nervous system growth-process is at its higher levels, while the different data on delivery modalities suggest that the stress of delivery is able to interfere on central oxygenation.

As NIRS is capable to evaluate the regional oxygenation, reflecting the effective brain oxygenation more accurately than systemic measurement, it is considered more adequate in the assessment of preterm infants whose cerebral auto-regulation system may be act inadequately (107). Indeed impairment in cerebral pressure auto-regulation is strongly related to an higher incidence of specific cerebral hemodynamic risks and there is no consistent relationship between blood pressure and cerebral pressure passivity (changes in blood pressure concordant with changes in CBF) (108,109). Soul *et al.* demonstrated that NIRS may provide a continuous measurements of cerebral perfusion in response to spontaneous blood pressure changes providing insights into the fluctuating nature of cerebral pressure passivity in the first days after birth and identifying infants at risk (109).

Considering H-I insult, a comparison between hypoxic-ischemic infants and an healthy group in terms of NIRS parameters showed that rSO2 in the healthy group was significantly higher than rSO2 in hypoxic-ischemic group, while there was no difference in SaO2 value between the two groups (110). These results probably reflect a different hemodynamic state, as a normal cerebral metabolism have been already achieved in the healthy group on the contrary of the hypoxic-ischemic which present a considerable impediment in circulation. In addition the study also demonstrated a consistent increase of HbO2 and decrease of Hb respectively in the healthy and hypoxic-ischemic group, testifying a great release of oxygen from the blood than the normal supply in case of hypoxia. Data have been shown also about the relation between CBV measured by NIRS and adverse outcome, as an increase in CBV on the first day of life is considered this a sensitive predictor of adverse outcome (111). At the same an increasing TOI suggests a risk of abnormal neurological outcome at one-year, this parameter is generally higher (more than 80.1%) in the group with an abnormal outcome, compared with control group (66.4%) and asphyxiated infant with positive outcome at one-year (74.7%), representing a marker of O2 brain delivery and energy failure in asphyxiated newborn infants (112).

At last, some investigators explored the ability of NIRS to provide a measure of fetal cerebral oxygenation both during the antepartum and intrapartum periods, since fetal heart rate monitoring, fetal pulse oximetry, and ultrasound testing have not been demonstrated to sufficiently reduce fetal/neonatal hypoxic-ischemic injury. *In vivo* data collected during the antepartum period showed a fetal cerebral oxygen saturation of 50-74% in healthy fetus (113), while fetal animal studies reported a significant reduction of CBF, CBV and oxygenation after cord occlusion despite a normal perfusion pressure and heart rate (114). On the other hand, NIRS parameters collected during intrapartum phase have been made by transvaginal placement of the optodes directly on the fetal head after rupture of membranes and sufficient cervical dilation. The collected parameters seemed to be significantly influenced by maternal contractions, during labour *in vivo* data showed a decrease in Hb, HbO2, CBF and fetal heart deceleration, even if a return of Hb to baseline is reported after contractions, these alterations can be considered physiological as they are not related to H-I insult in the fetus and the newborn (115,116). Currently NIRS is insufficiently developed to be used in labour and especially the collecting data phase need to further improvements.

To date, NIRS is not commonly performed, despite its several advantages and diagnostic possibilities. Indeed, NIRS still present some limitations that prevent its routine clinical use. Data on possible correlation between NIRS and monitoring parameters are still lacking, as well as data on possible changes in rSO2 in relation to gestational age. Some limitations are related to technical problems as the reproducibility of NIRS parameters, as they only referred to changes in the baseline curve, as the more adequate pathlength or the frequent evidence of movements or light artefacts (117,118). In this contest, biochemical markers measurement assumes more and more weight since also standard monitoring procedures, imaging techniques and NIRS are still lacking in detecting newborns at risk of hypoxic-ischemic insult and they may represent the more innovative preventive and diagnostic strategies in this field.

6. EXPERIMENTAL REPORTS SUPPORTING BRAIN DAMAGE MARKERS USEFULNESS

Based on literature data the gold standard of researches in perinatal medicine is "prevention", with the aim of improving our ability to detect fetuses, newborns and children at risk of brain injury at an earlier stage, when the window for therapeutic action is still open. Recent reports on experimental models and in humans show that the time-window for neuroprotection strategies is restricted to the first 6 hours after H-I insult. Therefore, the early identification of patients at risk of these developmental deficits and subsequent remedial interventions are of paramount importance to guarantee optimal development in these children. Although a normal life expectancy and quality of life might be assumed in patients with early normalization of their status, negative developmental outcome might not supervene until later in life.

6.1. Activin A: *In vitro* evidences for a H-I-related Regulation

Activin-A is a glycoprotein composed by two beta-A subunits, belonging to the transforming growth factor-beta (TGF-beta) superfamily of differentiation factors (119), and expressed in the CNS, where activin-A subunits and receptors are widely distributed, in both developing and mature mammalian brain (120).

A large body of evidence has been accumulated showing that brain lesions up-regulate the expression of activin A (121), and their temporal and spatial interplay seems to be crucial for the orchestration of postlesional restructuring. Studies employing models of acute brain injury strongly favor the notion that enhanced activin A expression represents a common response to acute neuronal damage of various origins. With respect to hypoxic/ischemic injury, using a unilateral model of hypoxic ischemic brain injury (double ligation of the right carotid artery of 21-day-old Wistar rats) is associated with a strong induction of activin beta-A subunit mRNA as early as 1 h after injury in the dentate gyrus of the non-ligated hemisphere, and 24 h after injury in the hippocampus, piriform cortex and amygdala on the non-ligated hemisphere. The expression of beta-A subunit was seen in the center of the infarcted region from five days after injury, however, the pattern of mRNA distribution strongly correlated with the expected distribution pattern for blood capillaries, thus suggesting a new role for activin A in the response to brain injury as local mediator of angiogenesis during the repair process (122).

Ribonuclease protection assay was used also to quantify the time course of the mRNA expression of activin beta-A subunit, following a 60-min H-I brain injury brain injury in 21-day-old Wister rats. Activin beta-A subunit mRNA level increased in the contra-lateral hemisphere 5 h after injury and returned to normal at 10 h post injury (123).

Whilst the notion that enhanced activin A expression represents a common response to acute neuronal damage of various origins is well assessed (124), the functional implications of enhanced activin A expression are not known at present. However, several experimental studies have shown that activin A has a beneficial role to neuronal recovery. Activin is known to support the survival of neurogenic cell lines and retinal neurons (125), and offering protection against neurotoxic damage in the cultures of midbrain dopaminergic neurons (126). Furthermore, activin A has been reported to enhance the survival of rat embryonic hippocampal neurons *in vitro* (127), to decrease ischemic brain injury in infant rats and to rescue striatal neurons against neurotoxic damage in rats (128).

6.2. S100B: *In vitro* evidences for a H-I-related Regulation

The glial protein S100 belongs to a family of calcium-binding proteins found as homo- or hetero-dimers of two different subunits (alpha and beta). Different combinations of the subunits make up the heterodimeric forms alpha–alpha, alpha-beta and beta-beta, types alpha-beta and beta-beta are described as S100B protein and are shown to be highly specific for nervous tissue.

In the nervous system, the protein appears to be most abundant in glial cells, although its presence in neuronal subpopulations has also been reported (129,130). In non-neural tissues, it is distributed widely in definite cell types, including melanocytes, Langerhans cells, chondrocytes, folliculostellate cells of the adenohypophysis, adrenal gland satellite cells, Leydig cells, and interdigitating reticulum cells (131), whereas adipose tissue constitutes a site of concentration for the protein comparable to the nervous tissue (132).

It has been shown that one hour of H-I significantly increased protein S100B release from rat brain slices, and such an increase is further enhanced by re-oxygenation of the ischemic slices (133). This pathway also occurs with other models of brain injury, since under severe metabolic stress conditions (withdrawal of oxygen, glucose and serum in cell cultures), astrocytes released the vast majority of the intracellular pool of S100B. This release occurred early and in the absence of significant cell death in the cultures, implying active, stress-triggered mechanism of S100B release during metabolic injury related to ischemia (134). When structural damage such as infarction occurs in the cytosol of glial and Schwann cells, S100B is released into the cerebrospinal fluid (CSF) and the blood (135,136) and, in human cerebrovascular diseases, a significant correlation has been reported between the plasma concentration of S100B protein and the volume of the cerebral infarct (137).

These findings have answered the question about the putative role of S100B in brain injury. S100B may play a dual role in the regulation of cell function, being beneficial to cells at low doses but detrimental at high doses (138). The fact that astroglia are able to produce S100B is of relevance in order to better understand the net role played by such a protein after brain injuries. Indeed, it is well known that glial (microglia and astroglia) cells are activated in the white matter (WM) aberrantly after chronic

cerebral hypoperfusion (139). This activation occurs in a manner that predicts the extent and severity of the subsequent WM damage, suggesting an important role of glial activation in the pathogenesis of WM lesions. In the susceptible WM, apoptosis of the oligodendroglia is induced with an up-regulation of inflammatory cytokines including TNF-alpha, and free radicals released from activated microglia and astroglia (140). In addition, the compromised blood–brain barrier (141) may allow the entry of macromolecules and other blood constituents such as proteases, immunoglobulins, complements, and cytokines into the perivascular WM tissues. astroglial activation exclusively. In studies using a neuronal and astroglia co-culture system, a high concentration of S100 protein up-regulated NO release from the astroglia, which was shown to be neurotoxic (137,142). Although the mechanism of responsible for this astroglial activation by a low concentration of S100 protein remains unclear, this protein is believed to be further activated by a positive feedback loop (143,144). It is postulated that these excessively activated astroglia may cause secondary tissue damage by the production of cytotoxic cytokines such as TNF-alpha, and COX2 and iNOS (145,146). Indeed, the delayed expansion of the cerebral infarction was accompanied by astroglial activation as well as by an increased tissue level of S100B protein in the peri-infarct area. Thus, the astroglial over-expression of S100B protein is considered to play a pivotal role in infarct expansion by causing alterations in the activities of multiple intracellular signaling pathways and the expression of various downstream proteins (147,148). To support this hypothesis are the data regarding the protective effect of arundic acid against astroglial activation and WM lesions during chronic cerebral hypoperfusion, by suppressing the increase in S100B content and, therefore, preventing excessive activation that may be harmful to neighboring neurons (147,149). Contrasting data have been recently reported by Ellis *et al.* (40), that reported that S100B protein is released from rat neonatal neurons, astrocytes, and microglia by *in vitro* trauma and that anti-S100B, able to bind and prevent the binding of S100B actions, increases trauma-induced delayed neuronal injury and negates the protective effect of exogenous S100B on neurons.

6.3. Adrenomedullin: *In vitro* evidences for a H-I-Related Regulation

AM is a C-amidated peptide belonging to the calcitonin gene-related peptide family (150). AM was first isolated from human pheochromocytoma by its capability to increase cyclic adenosine monophosphate production (151). Later, AM and its mRNA were found in many tissues from different species. The main proven function of AM is vasodilatation (151), although other actions have been reported, including neuromodulation (152) and inhibition of apoptosis (153,154). Expression of the AM gene is up-regulated by hypoxia (155,156) and inflammation (157), which are associated with neo-vascularization. Studies using AM gene knockout mice revealed that AM plays an important role in vascular formation in embryos (158-160).

In the CNS, AM was first found in the hypothalamus (161) but later its distribution was reported to be generalized in the rat CNS (162). The caudate-putamen was the only cerebral area where AM was found in the neuronal nuclei (162) and is one of the most sensitive brain areas to hypoxic damage. Indeed, AM is involved in response to hypoxia, at least in part by means of the transcription of the Hypoxia-Inducible Factor-1, which enhances AM expression and stabilizes AM mRNA (163). In the brain, it has been reported that ischaemic injury up-regulates AM expression in the cerebral cortex (164) and in the caudate-putamen (165) of the adult rat brain. Moreover, by exploiting DNA microarray technology to evaluate the brain genomic response of neonatal rat to hypoxia it was found that AM gene in neurons is up-regulated from 8 to 9 folds by hypoxia via HIF-1 (166).

In the CNS, where AM is mainly expressed in neurons and the endothelium (167), it is reported that transient ischemia boosted AM expression for more than 15 days (155). However, the role of augmented AM has remained unclear for inconsistent previous results: three studies reported neuro-protective effects of AM by demonstrating reduction of infarct size after transient ischemia (168-170), while one study detected exacerbation of infarction as a result of AM infusion (155).

The effects of AM on degenerative or regenerative processes in ischemic brain was test by using AM-transgenic (AM-Tg) mice that overproduce AM in the liver and performing middle cerebral artery occlusion for 20 minutes (20m-MCAO) to examine. It was found that: i) the infarct area and gliosis after 20m-MCAO was reduced in AM-Tg mice in association with suppression of leukocyte infiltration, oxidative stress and apoptosis in the ischemic core and that ii) vascular regeneration and subsequent neurogenesis were enhanced. These evidences and the finding that exogenous administration of AM in mice after 20m-MCAO also reduced the infarct area, and promoted vascular regeneration and functional recovery together suggests neuro-protective and vasculo-neuro-regenerative roles of AM (171).

6.4. NSE: evidences for a H-I-related regulation

NSE is a dimeric isoenzyme of the glycolytic enzyme enolase and derives from neuronal cytoplasm and neuron-endocrine cells: it occurs as gamma-gamma-enolase in neurons and as alpha-gamma-enolase in neuron-endocrine cells and in small lung cancer cells (172). NSE has a molecular weight of 78,000 Da and a biological half-life of 24 h, and into the CNS it is characterized by its consistent occurrence in the cytoplasm of mature neurons: immunoreactivity for NSE has been found in almost all paraneurons of both sensory and endocrine nature, suggesting that a unique system of intracellular energy metabolism may be shared by neurons and paraneurons (173). NSE could be traced by immunohistochemistry everywhere in the cerebral circulation after experimentally induced cerebral lesions in rats, and plasma concentrations correlated with events of neuronal NSE loss in focally ischemic neurons (174).

Several authors have shown that different cerebral diseases such as ischemic stroke, meningo-encephalitis and head injury cause an elevation in the

serum concentration of NSE (175,176). The serum levels correlated with the extent of brain damage and in some studies with the prognosis of the patients. However, it must be taken in mind that NSE also exists in platelets and in erythrocytes, and therefore hemolysis might produce a false rise in NSE (177) Consequently, an index of hemolysis should be determined before deciding whether or not to perform NSE measurement (178), since there appears to be an association between NSE and the degree of hemolysis (measured as free hemoglobin) after a short period of extracorporeal circulation (20 minutes), but not after extended periods, possibly indicating that release from the brain also contributed to the total concentration (177).

It was reported that NSE has neurotrophic activity (179), and that the synthetic peptide corresponding to the C-terminal portion of NSE (residues 404-433) also promotes the survival of neocortical neurons (180). The neurotrophic effects of NSE was also demonstrated on cultured mesencephalic and spinal neurons from rat embryo, since NSE promoted the survival of neurons not only in neocortical cultures but also in mesencephalic and spinal cord cultures in a low-oxygen atmosphere (181).

6.5. GFAP: Evidences for a H-I-Related Regulation

The interdependence of neurons on astrocytes and vice versa is crucial for the function of both types of cells in the CNS, especially during cerebral ischemic conditions (182). Astrocytes perform many functions which can have profound impact on the neurological consequences following cerebral ischemia (183). Moreover, the intimate relationship between astrocytes and neurons suggests that the fate of astrocytes can be crucial to the pathogenesis of ischemic injury. It has long been postulated that astrocytes are more resistant to hypoxic/ischemic challenge than neurons, however evidence has been accumulating that astrocytes not only do die but their death may even precede neuronal loss following cerebral ischemia (183-187).

GFAP is a monomeric intermediate filament protein of the astroglial skeleton with a molecular mass between 40 and 53 kDa. GFAP is found in the white and gray matter of the CNS and is considered brain specific (188,189). GFAP is released rapidly out of damaged brain and is up-regulated through astrogliosis (188,190,191). On this regard, Herrmann *et al* (190) demonstrated a continuous increase in serum GFAP from admission to the fourth day after ischemic stroke and GFAP values correlating to the size of the infarcted brain areas. In 2006, Foerch *et al* (192) showed that serum GFAP increased rapidly after intracerebral hemorrhage. Soon afterward, Vos *et al* (193) described an association between the severity of the initial brain injury after subarachnoid hemorrhage and s-GFAP taken on arrival at hospital. In patients with persistent middle cerebral artery occlusion, GFAP increased significantly compared with patients with normal sonographic findings and recanalization after thrombolysis resulted in a significant reduced increase (194). Finally, elevated GFAP concentrations in CSF have been described in various CNS diseases (138,195-197).

Levels of GFAP were assayed in the CSF of full-term infants between 12 and 48 h after birth. They were found to be increased 5-fold in infants after perinatal asphyxia compared with a reference group and to increase gradually in accordance with the severity of the neurological symptoms ranked as degree of HIE (198). In another study, GFAP was measured in CSF collected during the first 4 d of life from asphyxiated infants and compared to levels in infants without signs of perinatal asphyxia. Levels were significantly increased in the CSF of asphyxiated infants, and correlated significantly with other indicators of long-term prognosis and to neurological impairment at 1-y of age, or death before that time (151). When evaluated in the CSF of preterm infants, concentrations were found to be five times higher in preterm infants (n = 10) with an abnormal neonatal course and/or an abnormal neurological outcome than in healthy preterm infants. The positive predictive value of a GFAP higher than the 98th percentile of normal infants was 69%, while a GFAP level below this limit invariable predicted a good outcome (199).

7. CLINICAL DATA ON USEFULNESS OF BIOCHEMICAL SCREENING OF BRAIN DAMAGE

7.1. Activin A as a Brain Damage Marker in Infants complicated by H-I reperfusion Injury

With respect to human studies, the availability of a suitable assay developed in the last few years has made it possible to measure activin A concentrations after brain injury in humans, mainly occurring in the perinatal period. Indeed, umbilical cord activin A levels were evaluated in preterm newborns, and the diagnostic accuracy of their measurements to predict the occurrence of perinatal IVH. Activin A levels were significantly higher in preterm newborns developing IVH than in those who did not a follow up, and at the cut-off indicated by the ROC curve analysis activin A achieved a sensitivity of 100% and a specificity of 93% as a single marker for the prediction of IVH in preterm newborns (200).

In a longitudinal cohort study activin A levels were measured into the CSF collected at birth from healthy babies and from asphyxiated full-term newborns, that experienced hypoxic ischemic encephalopathy (HIE) within the first 7-days after birth. Briefly, full-term asphyxiated infants had increased CSF activin-A levels than healthy newborns, suggesting that hypoxia/asphyxia triggers activin A secretion. Furthermore, concentrations were higher in the asphyxiated infants who developed severe HIE than in those who did not or in controls, supporting the notion that elevated CSF activin A levels are reasonably a direct expression of CNS increased production. By the mean of activin A measurement, the early prediction of hypoxic ischemic brain lesions was possible before the appearance 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 (201).

More recently, activin A levels were measured in urine collected immediately after birth in asphyxiated full term newborns, and the diagnostic accuracy of their

measurements to predict the occurrence of perinatal encephalopathy was evaluated. Briefly, activin A levels in urine were significantly (P less than 0.0001) higher in asphyxiated newborns with moderate or severe HIE than in those with absent of mild HIE and controls. Moreover, an activin A concentration cut-off of more than 0.08 ng/L at first urination had a sensitivity of 83.3 % and a specificity of 100 % for predicting the development of moderate or severe HIE (202).

7.2. S100B As A Brain Damage Marker in Infants complicated by I-R Injury

The evidences that i) S100B is not affected by hemolysis, ii) it remains stable for several hours without the need for immediate analysis and, iii) its short half-life, together did S100B make measurements crucial in the emergency and intensive care settings (203). Given these characteristics, S100B has shown promise regarding its use as a possible serum marker for H-I brain damage. Moreover, as increased S100B concentration induced by brain H-I is believed to provide a biochemical information about the extent of the brain damage, either clarifying its release mechanism under ischemic conditions or understanding its advantages over other biochemical markers seems to be important for developing new therapeutic approaches against ischemia-induced neuronal damage.

CSF was the first of various biological fluids in which the role of S100B as a marker of active brain damage was shown (204,205). In perinatal medicine, measurements of S100B protein in CSF have been used to monitor infants affected by perinatal asphyxia and post-hemorrhagic ventricular dilatation brain damage during cardiac surgery. S100B concentrations 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 (206-208).

The idea to measure S100B into blood was based on the hypothesis that during active brain injury at least some of the S100B released from the damaged tissue could spread into the systemic circulation (209), also as a result of hemodynamic rearrangement of the blood brain barrier. Indeed, with respect to perinatal medicine, increased blood concentrations of S100B were detected 48 to 72 h before any clinical, laboratory, or ultrasound signs of cerebral bleeding (i.e. IVH) in preterm infants (210) HIE in full-term infants (211,212). In the latter study, Nagdyman *et al.* (136) reported that S100B protein concentrations in cord blood were already significantly higher in asphyxiated full-term infants suffering from birth asphyxia and HIE.

The same authors performed longitudinal S100B protein monitoring in peripheral blood and demonstrated a peak concentration of the protein 6 h after birth with a progressive decrease in S100B 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% (212). 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 (210,211). In asphyxiated full-term infants, an early increase in S100B was found to be predictive of HIE and subsequent adverse neurological outcomes (212).

S100B protein has also been used to monitor the occurrence of cerebral complications in preterm and term infants undergoing extracorporeal membrane oxygenation support for treatment of respiratory distress (213,214). S100B concentrations have been shown to be higher in IUGR fetuses with redistribution of fetal-placental blood flow, the so called “brain sparing effect”, and correlated with the degree of fetal hemodynamic impairment, as indicated by an altered middle cerebral artery Doppler pattern, whereas IUGR fetuses without “brain sparing effect” showed S100B concentrations similar to those of non-IUGR fetuses (215). On this regard, S100B was also measured in the blood of women whose pregnancies are complicated by IUGR and whose newborns develop IVH (216). At times before clinical, laboratory, and ultrasound patterns can identify risk of IVH, maternal S100B was higher in IUGR pregnancies complicated by IVH than in those that were not and in unaffected pregnancies. At a cut-off of 0.72 μg/L, sensitivity was 100% and specificity was 99.3% for prediction of IVH (216).

S100B is also assessable into urine fluid, and in the urine of healthy preterm and term newborns its concentrations correlate with gestational age at sampling, offering a normality reference curve (217). Moreover, urine S100B concentrations at birth were significantly higher in preterm newborns who later developed cerebral bleeding and/or brain damage at a stage when all routine clinical, laboratory, and ultrasound investigations were still silent. Longitudinal monitoring of urine S100B concentrations showed a progressive increase in the concentration of the protein 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% (218). The measurement of S100B in urine has been also used as an early indicator of risk of neonatal death. Indeed, in a cross-sectional study using urine obtained from 165 preterm newborns, of whom 11 suffered neonatal death within the first week, 121 displayed no overt neurological syndrome, and 33 suffered neonatal hypoxia and IVH but not ominous outcome, S100B concentrations were 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% (219).

Finally, the clinical usefulness of urine S100B measurement for early detection of post-asphyxia brain damage was evaluated in asphyxiated full-term newborns (219). The concentrations of S100B protein in urine were higher in samples collected newborns with abnormal neurological findings on follow-up than in samples from those without 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 (219).

7.3. AM as a Brain Damage Marker in Infants complicated by H-I Reperfusion Injury

Boldt *et al.* (220) reported measurements of AM concentrations in umbilical arterial and venous blood of infants born vaginally and by caesarean section. They found higher concentrations in infants born vaginally, and there was a linear correlation between pH and AM concentrations. Based on these observations, they indicate that their findings support a physiological role for AM in vascular adaptation after birth: stress of labor would activate the fetal secretion of the peptide with the aim to increase pulmonary blood flow, since it is AM is a potent dilator of the pulmonary circulation (221).

With respect to the putative function in the fetus, it was also suggested that AM may play a role in fetal cardiovascular adaptation, since infants affected by IUGR, a well known disease characterized to a poor fetal tissues perfusion, share higher AM concentration that unaffected newborns (222). On the contrary, failed to detect significant different AM levels in IUGR newborns (223). With respect to pregnant tissues, in placentas collected from term pregnancies complicated by birth asphyxia, AM mRNA was were present in asphyxiated newborn infants with severe HIE compared with patients with mild or no HIE (224).

AM was therefore measured at birth in infants with prenatal asphyxia who developed IVH, and levels were compared to those circulating in newborns with asphyxia without IVH, and healthy controls. Concentration, measured at 12 h from birth, were significantly higher in neonates with asphyxia that developed IVH, than in the remaining patients. Those data allowed Authors to conclude that AM may participate in the loss of cerebral vascular autoregulation in response to hypoxia and could be useful to discriminate, among newborns at risk, those with an adverse neurological outcome (225).

7.4. NSE as a Brain Damage Marker in Infants complicated by H-I Reperfusion Injury

Physiologically, NSE is present only in negligible amounts in the peripheral blood. Tumor cells in APUDoma, neuroblastoma, and small cell carcinoma of the lung are capable of producing NSE and are usually accompanied by elevated serum titers. For this reason, NSE has been established as a diagnostic and prognostic serum marker in the clinical management of these neoplasms (226). Recent studies showing an increase in CSF and serum NSE levels after ischemic stroke, IVH, and brain injury support the contention that NSE may also be a sensitive and quantitative marker of parenchymal brain injury (227,228).

NSE levels were measured both in serum and CSF in the immediate post-asphyxia period in term newborn infants: NSE in CSF correlated with the degree of asphyxia damage and were significantly increased in the CSF of infants that developed HIE compared with control infants (227). Then, NSE was measured into CSF at 12 and 72 hours of life in asphyxiated infants that were studied with serial neurological examination, cranial ultrasonography, and neurological follow-up. It was found that NSE concentrations were related to the degree of neonatal HIE, and correlated with adverse outcome (death or cerebral palsy at 1 year) (228). These data are in agreement with an other study by Blennow *et al.* (207), that evaluated NSE levels in the CSF collected during the first 4 d of life from asphyxiated infants. Briefly, they found that NSE concentrations were significantly increased in the CSF of asphyxiated infants, and concentrated significantly with other indicators of long-term prognosis and to neurological impairment at 1 y of age, or death before that time.

When CSF NSE levels were investigated along with cranial ultrasonography, magnetic resonance imaging (MRI), and electroencephalography (EEG) for predicting the clinical state and neurological outcome in asphyxiated term newborns, it was shown that: i) NSE concentrations in babies in the whole HIE group were higher than babies in the "no encephalopathy" group, ii) levels were increased in newborns with HIE grade 2 and 3 than both the no encephalopathy and HIE grade 1 groups. Moreover, the findings of cranial MRI, EEG, and CSF NSE levels were found to be predictive of outcome of H-I brain damage in asphyxiated newborns, and this predictivity would increase with the combination of these diagnostic parameters (229).

NSE was evaluated in the serum collected between 4 and 48 h and 5-7 days after birth from asphyxiated full-term newborn infants who developed symptoms and signs of HIE (Group 1) and in full-term newborn infants with normal physical examination. In the newborns with asphyxia serum NSE levels were significantly increased, and higher in patients with stage III HIE than in those with stages II and I. The sensitivity and specificity values of serum NSE as a predictor of HIE of moderate or severe degree were 79 and 70%, respectively, and as a predictor of poor outcome were calculated as 84 and 70%, respectively (230). These data were not confirmed by Nagdyman *et al.*, (212,231) since they failed to retrieve a correlation between serum NSE and the HIE degree.

8. BRAIN DERIVED PROTEIN AND THE HUMAN MILK: AN EVOLUTIONARY STRATEGY?

Breast milk provides the primary source of nutrition for newborns and mainly contains substances that have a critical role in the growth and development of the newborn.

The exact composition of breast milk varies from day to day, with the maturation from colostrum to mature milk and also depending on food consumption and environment, foremilk is watery, low in fat and high in carbohydrates relative to the creamier hindmilk which is released as the feed progresses (232). Human milk contains

0.8-0.9% protein, 3-5% fat, 6.9-7.2% carbohydrates and 0.2% minerals. Carbohydrates are mainly lactose, while the principal proteins are casein, alpha-lactalbumin, lactoferrin, IgA, lysozyme and serum albumin. Non-protein nitrogen-containing compounds, making up 25% of the milk's nitrogen, include urea, uric acid, creatine, creatinine, amino acids and nucleotides (233). In addition to the appropriate amounts of carbohydrate, protein and fat, breast milk also provides vitamins, minerals, digestive enzymes, hormones, and, mainly, antibodies and lymphocytes from the mother. These molecules are biologically active, as they participate in multiple physiological processes, including modulation of gastrointestinal functions, microbial growth control and immunoregulation. These bioactive compounds include peptide hormones and growth factors such as insulin, transferrin, lactoferrin, epidermal growth factor (EGF), transforming growth factor (TGF), nerve growth factor (NGF), and insulin-like growth factors I and II (IGF-I and IGF-II) (234). The concentrations of these peptides are generally higher in colostrum than in milk and the limited protease activity in the gastrointestinal tract of neonates, and the existence of protease inhibitors in milk, allows these peptides surviving to the gastrointestinal digestion, be absorbed through the gastrointestinal tract and appear in plasma. Among these peptides, some biological molecules involved in the regulation of newborn growth, including brain development, have been recognized.

Many works analyzed the influence of early diet , mainly lipids composition, on the development of newborn, demonstrating that malnutrition cold affect the maturity of the brain. This may be due to the lack of essential fatty acids, and will particularly involve premature babies born at a time when cell membrane development is especially vulnerable. Breast milk contains docosahexaenoic acid (DHA), arachidonic acid (AA), linoleic acid (LA) and long-chain polyunsaturated fatty acids (LCPUFAs) which are suggested to be essential for normal brain development, and often absent or in short supply in formula feeds (235). Knowledge of the importance of these molecules in neurodevelopment was originally obtained from animal studies. These fatty acids are found in high concentrations in breast milk and are rapidly accreted in brain during the first postnatal year in animal and human infants where they may act in different ways. LCPUFA may enhance intellectual development in breast-fed children, while DHA seem to be involved in visual and neural systems maturation (236). Esterified poplyunsaturated fatty acids act in cellular membranes, in signal transduction, in neurotransmission, and in the formation of lipid rafts, on the other hand, nonesterified polyunsaturated fatty acids can modulate gene expression and ion channel activities, thus becoming neuroprotective agents. The conversion of linoleic acid and alpha-linolenic acid into ARA and DHA would enhance visual and cognitive development (236,237). Moreover, these molecules may have a fundamental role in synapses formation (238) and maturation of specific part of brain, such as brainstem. Infants fed breast milk have faster brainstem maturation, compared with infants fed formula and this effect may be attributable to the constituent composition of breast milk, compared with synthetic formulas (239). At last, also the concentration of sialic acid in brain gangliosides and glycoproteins has been linked to learning ability in animal studies. Human milk is a rich source of sialic acid-containing oligosaccharides and higher brain ganglioside and glycoprotein sialic acid concentrations in infants fed human milk suggests increased synaptogenesis and differences in neurodevelopment (240). Overall, these emerging data suggest that human milk may especially benefit the premature infant (241), but also on term infants as some factors couls exert a neuroprotective action.

On this regard, other molecules habe been investigated to evaluate the possible protective and preventive role of human breast milk on neurodevelopment. We recently investigated the presence and the role of S100B in human milk (242). The presence of a calcium-binding protein in a biological fluid such as milk, in which calcium is abundant, is not surprising, in the light of the consideration that other calcium-binding proteins (e.g. alpha-lactalbumin, calmodulin, osteocalcin) have already been detected in milk. It is interesting that the concentration of S100B in milk is markedly higher than that observed in other biological fluids such as cord blood, peripheral blood, urine, cerebrospinal fluid and amniotic fluid (217, 243-245). This discovery is important the suggest a putative role of S100B as cytokine with neurotrophic effect and breast milk exerts a stimulating effects on brain maturation. More detailed information on the fate of the S100B molecule in the gastrointestinal tract is also needed to support the possibility that S100B may participate in the nutritional effects of milk, including a role in brain development. However, a trophic role for S100B in milk would not be surprising, given that human breast milk is known to contain a variety of substances that may actively influence the growth and development of the infant, including hormones, growth factors and cytokines. Human milk is known to contain cell types expressing S100B protein, including mammary epithelial cells and lymphocytes which may reasonably be supposed to be the sources of S100B mRNA and of S100B protein. It is also reasonable to suppose that a significant part of the S100B protein present in milk is secreted by mammary epithelial cells, which are known to express the protein (131), taking into consideration the ascertained extracellular role of the protein and its high concentration in human milk. However, whereas fat cells are a site of concentration for S100B (132) lipids are known to be present in milk only as membrane- surrounded globules secreted by mammary epithelial cells (246). S100B has been found in all type of human milk, colostrum, transition milk and mature milk, even if its concentration was lowest in colostrum and then increases progressively (247). An important correlation was found between S100B concentration and gestational age. It was also found in low dose in milk-formulae milk. There are also differences between the human milk and the milk-formulae milk that are probably related to the routinely preparation procedures (248). On the other hand, to date there are no data on the absorption of S100B in maternal milk by infants and, therefore, on the effect of a potential contribution of exogenous S100B to measurements of the protein in the biological fluids of infants.

AM was also investigated even if the number of studies is very small. *In vivo* studies on animals

demonstrated the presence of AM both in bovine and mouse milk. Pio *et al.* demonstrated a concentration of AM in fresh bovine milk of 73.5 6 3.8 pg/mL and also in different milk products (whole milk and skim milk) they found a comparable levels to those found in fresh bovine milk (249). Also in the case of the bovine milk-based infant formulas, the levels were similar to those found in fresh bovine milk (59.5 +/- 3.4 and 57.8 +/- 6.5 pg/mL) (249). Jahnke *et al.* performed Western blot for detection of AM on mouse milk and it showed evidence of three AM forms, the fully processed AM form, the intermediate and the unprocessed form, and whose bands presented greater in intensity (250).

Considering human breat milk, Ohta *et al.* analyzed breast milk samples of women during postpartum days 1–8 and AM was only detectable in 21% of samples at an avarage concentrations of 7.2 +/- 5.8 pmol/L and a range between 2.7-20.7 pmol/L (251). This finding appears to conflict with that of Pio *et al.,* who measured AM by radioimmunoasssay (RIA) in milk samples from three healthy lactating women. They reported that AM concentrations in breast milk were higher (23–67 pmol/L), even if the analysed population was very small (249). Recently Cekmen *et al.* investigated the concentrations of AM in colostrum and 30th day-milk in healthy women. AM levels resulted 16.59 +/- 1.24 pg/mL in colostrum, while decreasing levels were subsequently showed in the 30th day-milk of same patients (12.18 +/- 1.48 pg/mL) (252). Overall, all the reported investigations on human breast milk have been carried on a limited population limited to ensure a certain role of AM in human milk, but the results are encouraging and more researchers are needed.

Regarding activin A, only one study at the moment evaluated its presence and possible role in human breast milk. Indeed, since human breast is able to produce activin A, we evaluate whether it was measurable in breast milk of women during lactation. Activin A was measured in milk samples collected at 3, 5 and 30 days after delivery, and while no significant different concentrations between the third and the fifth day after delivery was found, activin A concentrations after 1 month of lactation was significantly decreased (253). Furthermore, no difference of activin A concentration between the whole and the skim milk or between spontaneous delivery and cesarean section was found (253). The evidence that activin A is present in human milk in high concentrations in the first week of lactation, while decrease after a month suggesting a possible role as growth factors in human milk.

On the other hand, the presence of these molecules in human breast milk, suggest also their fundamental role on breast maturation and differentiation. The development of the mammary gland proceeds in different phases of morphological and functional differentiation under the influence of several hormones, such as estrogen, progesterone, placental lactogen, prolactin, and oxytocin. The differentiation of secretory epithelial cells represent a critical aspect in the reproductive cycle of female. Indeed, a pronounced ductal growth occurs at the onset of puberty while the alveolar proliferation occurs during pregnancy with a terminal differentiation at the end of gestation and the onset of milk secretion at parturition. On this regard, the action of activin A and adrenomedullin have been mainly investigated. An *in vivo* animal study demonstrated that the inactivation of activin/inhibin beta-B genes results in incomplete mammary development and failure of lactation. Besides, ductal eleongation is incomplete and morphogenesis of secretory alveolar ducts is reduced. We can supposed that these alterations reflects a perturbance in growth regulation due to the lack of stimuli from mammary stroma (254). Indeed, it is not surprising that activin and inhibin may act as paracrine and autocrine growth factors in mammary glands, both in physiological and pathological circumstances (255).

Considering AM, in light of previous reports suggesting a role for AM in hormone secretion, cell growth and cAMP regulation, the involvement of AM and its receptor (AM-R) in the mammary gland have been investigated. Welsch *et al.* (256) showed that AM immunopositivity is present in both lactating and non-lactating mammary glands. Identification of AM immunopositivity and of electron dense secretory granules in basal parts of epithelial cells suggests that AM may be secreted preferentially into the bloodstream, acting as an endocrine signal. Moreover, the localization of both AM ligand and receptor to ductal epithelial cells and AM-R without the ligand to the periductal and fat pad fibroblasts suggests possible autocrine and paracrine involvement respectively.

Overall, the presence of these substances in mammary gland does not necessarily imply a physiologic role for that entities in human brest milk. The real biologic significance is difficult to establish and requires the demonstration of an effect in the children in response to the exposure to the substance in milk as well as the effect in response to the removal of the substance from milk. However, it is possible to speculate that the presence of the active peptide AM, S100B, activin A in milk could have some direct impact in the development of the neonate due to the several physiological activities that have been associated to them and a nueroprotective role may be proposed.

9. CONCLUSIONS

There is evidence from the emerging useful collaboration among researchers of several disciplines (i.e. laboratory, intensive care medicine, pediatric, neuroscience, heart surgery, cardiology etc.) that the bulk of studies on potential usefulness of biochemical markers for early brain injury detection is growing day by day. From a clinical point of view, the ambitious aim is to satisfy the constant need for practical and sensitive markers able to identify patients at higher risk, as early as possible, in order to take immediate preventive or therapeutic measures and to include such markers in evidence-based guidelines. In other words, the measurement of biochemical markers in biological fluids need to by satisfy the following clinical criteria: i) to constitute an alternative and direct indicator of CNS damage when clinical and radiological

assessments are still silent, ii) to provide a quantitative indicator of the extent of brain lesions and iii) to offer useful information on the effectiveness of the therapeutic strategies performed. Nonetheless, additional criteria are requested for such markers, from a laboratory point of view, that are strictly linked one each other: i) an assay simple to perform measurements with a good reproducibility, sensitivity and specificity, ii) the possibility to be measurable assessed in a variety of biological fluids in order to reduce newborns and children stress due to sampling modalities, iii) possible use in longitudinal monitoring, iv) low costs , and v) well-established and validated use as an early and quantitative marker of brain lesions/damage. Taken together these queries can be all defined as an optimality concept for biochemical markers inclusion in evidence-based guidelines. At present, the conclusion of the findings, on different biochemical markers, herein reported suggest that only few have been assessed under all these point 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 laboratory performance differences that need to be elucidated. For example, one of the major point consists on the possibility of the assessment in different biological fluids. In this regard, S100B protein is the only one previously reported to be detectable in urine and saliva fluids, whilst, to our knowledge, no data are reported for other markers (AM, GFAP and NSE). The finding could be of relevance in order to perform longitudinal monitoring, especially useful under severe intensive care conditions such as extremely preterm newborns. Furthermore, reference curves for different biological fluids are not available for all markers.

Nevertheless, it should be highlighted that S100B and NSE monitoring during CPB could not be reliable since their assessment has been shown to be affected by CPB procedure modalities. This does not hold for Activin A and AM that seem to be more indicated for this purpose. Another criteria, that has to be taken into account, regards the reproducibility and the results output: results can be obtained within 6 hours for markers assayed by ELISA technique, whilst different LIAISON technique offers S100B result within 2 hours.

Last but not least is the cost for each sample: to our knowledge there are no significant differences among different techniques. It is noteworthy that 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, bearing in mind that the gold standard consists on detecting brain injury at an earlier stage, when the window for therapeutic action is still open, this preventive strategy can not be reached by a close interaction among several specialties involved in the management of high risk newborns and children.

10. ACKNOWLEDGEMENT

This work was partially supported by Grants COFIN/MIUR 2005 and from “Let’s Improve Prenatal Life” and “Stella Cometa” Foundations.

11. REFERENCES

1. Volpe J: Brain injury in the premature infant: overview of clinical aspects, neuropathology, and pathogenesis. *Semin Pediatr Neurol* 5:135-151 (1998)

2. Ingebrigtsen T and B. Romner: Biochemical serum markers for brain damage: a short review with emphasis on clinical utility in mild head injury. *Restor Neurol Neurosci* 21:171-176 (2003)

3. Hughes PE, T. Alexi, M. Walton, C. E. Williams, M. Dragunow, R. G. Clark, P. D. Gluckman: Activity and injury-dependent expression of inducible transcription factors, growth factors and apoptosis-related genes within the central nervous system. *Prog Neurobiol* 57:421-450 (1999)

4. Visser GH: Why does perinatal morbidity increase. *J Perinat Med* 22:28-34 (1994)

5. Hagberg B, G. Hagberg, I. Olow, L. von Wendt: The changing panorama of cerebral palsy in Sweden. VII. Prevalence and origin in the birth year period 1987-90. *Acta Paediatr* 85:954-960 (1996)

6. Botero D, F. Lifshitz: Intrauterine growth retardation and long-term effects on growth. *Curr Opin Pediatr* 11:340-347 (1999)

7. Inder TE, J. J. Volpe: Mechanisms of perinatal brain injury. *Semin Neonatol* 5:3-16 (2000)

8. Freeman JM, K. B. Nelson: Intrapartum asphyxia and cerebral palsy. *Pediatrics* 82:240-249 (1988)

9. Hughes I, R. Newton: Genetic aspects of cerebral palsy. *Dev Med Child Neurol* 34:80-86 (1992)

10. Hagberg B, G. Hagberg, E. Beckung, P. Uvebrant: Changing panorama of cerebral palsy in Sweden. VIII. Prevalence and origin in the birth year period 1991-94. *Acta Paediatr* 90:271-277 (2001)

11. Alvarez-Diaz A, E. Hilario, F. Goni de Cerio, A. Soler, F. J. Alvarez-Diaz: Hypoxic-ischemic injury in the immature brain--key vascular and cellular players. *Neonatology* 92:227-235 (2007)

12. Walton M, B. Connor, P. Lawlor, D. Young, E. Sirimanne, P Gluckman, G. Cole, M. Dragunow: Neuronal death and survival in two models of hypoxic-ischemic brain damage. *Brain Res Rev* 29:137-168 (1999)

13. Ferriero DM: Neonatal brain injury. *N Engl J Med* 351:1985-1995 (2004)

14. Sugawara T, M. Fujimura, N. Noshita, G. W. Kim, A. Saito, T. Hayashi, P. Narasimhan, C. M. Maier, P. H. Chan: Neuronal death/survival signaling pathways in cerebral ischemia. *NeuroRx* 1:17-25 (2004)

15. Lorek A, Y. Takei, E. B. Cady, J. S. Wyatt, J. Penrice, D. A. Edwards, D. Peebles, M. Wylezinska, H. Owen-Reece, V. Kirkbride: Delayed ("secondary") cerebral energy failure after acute hypoxia-ischemia in the newborn piglet: continuous 48-hour studies by phosphorus magnetic resonance spectroscopy. *Pediatr Res* 36:699-706 (1994)

16. Back SA, N. Ling Luo, N. S. Borenstein, J. M. Levine, J. J. Volpe, H. C. Kinney: Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. *J Neurosci* 21:1302-1312 (2001)

17. Hilario E, M. C. Rey-Santano, F. Goni-de-Cerio, F. J. Alvarez, E. Gastiasoro, V. E. Mielgo, A. Caballero, A. Soler, S. Gomez-Urquijo, A. Alvarez: Cerebral blood flow and morphological changes after hypoxic-ischaemic injury in preterm lambs. *Acta Paediatr* 94:903-911 (2005)

18. Volpe JJ: Brain injury in the premature infant--current concepts. *Prev Med* 23:638-645 (1994)

19. Rivkin MJ: Hypoxic-ischemic brain injury in the term newborn. Neuropathology, clinical aspects, and neuroimaging. *Clin Perinatol* 24:607-625 (1997)

20. Volpe JJ: Neurobiology of periventricular leukomalacia in the premature infant. *Pediatr Res* 50:553-562 (2001)

21. du Plessis AJ, J. J. Volpe: Perinatal brain injury in the preterm and term newborn. *Curr Opin Neurol* 15:151-157 (2002)

22. Berger R, Y. Garnier: Perinatal brain injury. *J Perinat Med* 28:261-285 (2000)

23. Johnston MV, W. H. Trescher, A. Ishida, W. Nakajima: Neurobiology of hypoxic-ischemic injury in the developing brain. *Pediatr Res* 49:735-741 (2001)

24. Halterman MW, C. C. Miller, H. J. Federoff: Hypoxia-inducible factor-1alpha mediates hypoxia-induced delayed neuronal death that involves p53. *J Neurosci* 19:6818-6824 (1999)

25. Droge W: Free radicals in the physiological control of cell function. *Physiol Rev* 82:47-95 (2002)

26. Jensen A, Y. Garnier, J. Middelanis, R. Berger: Perinatal brain damage-from pathophysiology to prevention. *Eur J Obstet Gynecol Reprod Biol* 110:S70-S79 (2003)

27. Buonocore G, S. Perrone: Biomarkers of hypoxic brain injury in the neonate. *Clin Perinatol* 31:107-116 (2004)

28. Fern R, T. Moller: Rapid ischemic cell death in immature oligodendrocytes: a fatal glutamate release feedback loop. *J Neurosci* 20:34-42 (2000)

29. Palmer C, J. Towfighi, R. L. Roberts, D. F. Heitjan: Allopurinol administered after inducing hypoxia-ischemia reduces brain injury in 7-day-old rats. *Pediatr Res* 33:405-411 (1993)

30. Hamada Y, T. Hayakawa, H. Hattori, H. Mikawa: Inhibitor of nitric oxide synthesis reduces hypoxic-ischemic brain damage in the neonatal rat. *Pediatr Res* 35:10-14 (1994)

31. Patkai J, B. Mesples, M. A. Dommergues, G. Fromont, E. M. Thornton, J. C. Renauld, P. Evrard, P. Gressens: Deleterious effects of IL-9-activated mast cells and neuroprotection by antihistamine drugs in the developing mouse brain. *Pediatr Res* 50:222-230 (2001)

32. Kristian T: Metabolic stages, mitochondria and calcium in hypoxic/ischemic brain damage. *Cell Calcium* 36:221-233 (2004)

33. Englund E: Neuropathology of white matter lesions in vascular cognitive impairment. *Cerebrovasc Dis* 13:11-15 (2002)

34. Counsell SJ, M. A. Rutherford, F. M. Cowan, D. A. Edwards: Magnetic resonance imaging of preterm brain injury. *Arch Dis Child Fetal Neonatal Ed* 88:F269-F274 (2003)

35. Peterson BS: Brain imaging studies of the anatomical and functional consequences of preterm birth for human brain development. *Ann N Y Acad Sci* 1008:219-237 (2003)

36. McQuillen PB, D. M. Ferriero: Selective vulnerability in the developing central nervous system. *Pediatr Neurol* 30:227-235 (2004)

37. Russ A, I. L. Hand: Preterm brain injury: imaging and neurodevelopmental outcome. *Am J Perinatol* 21:167-172 (2004)

38. Perlman JM: Intrapartum hypoxic-ischemic cerebral injury and subsequent cerebral palsy: medicolegal issues. *Pediatrics* 99:851-859 (1997)

39. Wyatt JS, D. A. Edwards, D. Azzopardi, O. E. Reynolds: Magnetic resonance and near infrared spectroscopy for investigation of perinatal hypoxic-ischaemic brain injury. *Arch Dis Child* 64:953-963 (1989)

40. Ellis EF, K. A. Willoughby, S. A. Sparks, T. Chen: S100B protein is released from rat neonatal neurons, astrocytes, and microglia by in vitro trauma and anti-S100 increases trauma-induced delayed neuronal injury and negates the protective effect of exogenous S100B on neurons. *J Neurochem* 101:1463-1470 (2007)

41. Adami C, R. Bianchi, G. Pula, R. Donato: S100B-stimulated NO production by BV-2 microglia is independent of RAGE transducing activity but dependent on RAGE extracellular domain. *Biochim Biophys Acta* 1742:169-177 (2004)

42. Tasaka K, K. Kasahara, N. Masumoto, J. Mizuki, H. Kurachi, A. Miyake, O. Tanizawa: Activin A increases cytosolic free calcium concentration in rat pituitary somatotropes. *Biochem Biophys Res Commun* 185:974-980 (1992)

43. Jauch EC, C. Lindsell, J. Broderick, S. C. Fagan, B. C. Tilley, S. R. Levine: Association of serial biochemical markers with acute ischemic stroke: the National Institute of Neurological Disorders and Stroke recombinant tissue plasminogen activator Stroke Study. *Stroke* 37:2508-2513 (2006)

44. Grow J, J. Barks: Pathogenesis of hypoxic-ischemic cerebral injury in the term infant: current concepts. *Clin Perinatol* 29:585-602 (2002)

45. Shu-Meil J, X. Jian-Mei, W. Chuan, S. Su-Wen, H. Rui-Rong: Adrenomedullin reduces intracellular calcium concentration in cultured hippocampal neurons. *Sheng Li Xue Bao* 57:340-345 (2005)

46. Roth SC, J. Baudin, E. B. Cady, K. Johal, J. P. Townsend, J. S. Wyatt, O. E. Reynolds, A. L. Stewart: Relation of deranged neonatal cerebral oxidative metabolism with neurodevelopmental outcome and head circumference at 4 years. *Dev Med Child Neurol* 39:718-725 (1997)

47. Palmer C: Hypoxic-ischemic encephalopathy. Therapeutic approaches against microvascular injury, and role of neutrophils, PAF, and free radicals. *Clin Perinatol* 22:481-517 (1995)

48. Magistretti PJ, L. Pellerin, D. L. Rothman, R. G. Shulman: Energy on demand. *Science* 283:496-497 (1999)

49. Choi DW: Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1:623-634 (1988)

50. Minc-Golomb D, Y. Levy, N. Kleinberger, M. Schramm: D-[3H]aspartate release from hippocampus slices studied in a multiwell system: controlling factors and postnatal development of release. *Brain Res* 402:255-263 (1987)

51. Hagberg H, P. Andersson, I. Kjellmer, K. Thiringer, M. Thordstein: Extracellular overflow of glutamate, aspartate, GABA and taurine in the cortex and basal ganglia of fetal lambs during hypoxia-ischemia. *Neurosci Lett* 78:311-317 (1987)

52. Danbolt NC: Glutamate uptake. *Prog Neurobiol* 65(1):1-105 (2001)

53. Delivoria-Papadopoulos M, O. P. Mishra**:** Cellular mechanisms of hypoxic injury in the developing brain. *Brain Res Bull* 48:233-238 (1999)

54. Fox K, B. L. Schlaggar, S. Glazewski, D. D. O'Leary: Glutamate receptor blockade at cortical synapses disrupts development of thalamocortical and columnar organization in somatosensory cortex. *Proc Natl Acad Sci U S A* 93:5584-5589 (1996)

55. Sibson NR, J. Shen, G. F. Mason, D. L. Rothman, K. L. Behar, R. G. Shulman: Functional energy metabolism: in vivo 13C-NMR spectroscopy evidence for coupling of cerebral glucose consumption and glutamatergic neuronalactivity. *Dev Neurosci* 20:321-330 (1998)

56. Jabaudon D, M. Scanziani, B. H. Gähwiler, U. Gerber: Acute decrease in net glutamate uptake during energy deprivation. *Proc Natl Acad Sci U S A* 97:5610-5615 (2000)

57. Tao F, S. D. Lu, L. M. Zhang, Y. L. Huang, F. T. Sun: Role of excitatory amino acid transporter 1 in neonatal rat neuronal damage induced by hypoxia-ischemia. *Neuroscience* 102:503-513 (2001)

58. Levene MI, N. A. Gibson, A. C. Fenton, E. Papathoma, D. Barnett: The use of a calcium-channel blocker, nicardipine, for severely asphyxiated newborn infants. *Dev Med Child Neurol* 32:567-574 (1990)

59. Mishra OP, M. Delivoria-Papadopoulos: Lipid peroxidation in developing fetal guinea pig brain during normoxia and hypoxia. *Brain Res Dev Brain Res* 45:129-135 (1989)

60. Crawford MA, A. J. Sinclair: Nutritional influences in the evolution of mammalian brain. In: Lipids, malnutrition & the developing brain. *Ciba Found Symp* 1971, :267-92.:267-292.

61. Maulik D, Y. Numagami, T. Ohnishi, O. P. Mishra, M. Delivoria-Papadopoulos: Direct measurement of oxygen free radicals during in utero hypoxia in the fetal guinea pig brain. *Brain Res* 798:166-172 (1998)

62. Khan YJ, S. M. Black: Developmental changes in murine brain antioxidant enzymes. *Pediatr Res* 54:77-82 (2003)

63. Ferriero DM: Oxidant mechanisms in neonatal hypoxia-ischemia. *Dev Neurosci* 23:198-202 (2001)

64. van Bel F, C. Roman, R. J. Klautz, D. F. Teitel, A. M. Rudolph: Relationship between brain blood flow and carotid arterial flow in the sheep fetus. *Pediatr Res* 35:329-333 (1994)

65. Ogihara T, K. Hirano, H. Ogihara, K. Misaki, M. Hiroi, T. Morinobu, K. Han-Suk, S. Ogawa, R. Ban, M. Hasegawa, H. Tamai: Non-protein-bound transition metals and hydroxyl radical generation in cerebrospinal fluid of newborn infants with hypoxic ischemic encephalopathy. *Pediatr Res* 53:594-599 (2003)

66. Subbarao KV, J. S. Richardson: Iron-dependent peroxidation of rat brain: a regional study. *J Neurosci Res* 26:224-232 (1990)

67. Almli LM, S. E. Hamrick, A. A. Koshy, M. G. Tauber, D. M. Ferriero: Multiple pathways of neuroprotection against oxidative stress and excitotoxic injury in immature

primary hippocampal neurons. *Brain Res Dev Brain Res* 132:121-129 (2001)

68 Sarco DP, J. Becker, C. Palmer, A. R. Sheldon, D. M. Ferriero: The neuroprotective effect of deferoxamine in the hypoxic-ischemic immature mouse brain. *Neurosci Lett* 282:113-116 (2000)

69. McLean CW, O. Mirochnitchenko, C. P. Claus, L. J. Noble-Haeusslein, D. M. Ferriero: Overexpression of glutathione peroxidase protects immature murine neurons from oxidative stress. *Dev Neurosci* 27:169-175 (2005)

70. Fullerton HJ, H. J. Fullerton, J. S. Ditelberg, S. F. Chen, D. P. Sarco, P. H. Chan, C. J. Epstein, D. M. Ferriero: Copper/zinc superoxide dismutase transgenic brain accumulates hydrogen peroxide after perinatal hypoxia ischemia. *Ann Neurol* 44:357-364 (1998)

71. Yue TL, J. L. Gu, P. G. Lysko, H. Y. Cheng, F. C. Barone, G. Feuerstein**:** Neuroprotective effects of phenyl-t-butyl-nitrone in gerbil global brain ischemia and in cultured rat cerebellar neurons. *Brain Res* 574:193-197 (1992)

72. Cao X, J. W. Phillis: alpha-Phenyl-tert-butyl-nitrone reduces cortical infarct and edema in rats subjected to focal ischemia. *Brain Res* 644:267-272 (1994)

73. Sorrentino DF, K. I. Fritz, S. H. Haider, N. Parikh, M. Delivoria-Papadopoulos, Om P. Mishra: Nitric oxide-mediated modification of the glycine binding site of the NMDA receptor during hypoxia in the cerebral cortex of the newborn piglet. *Neurochem Res* 29:455-459 (2004)

74. Ferriero DM, D. M. Holtzman, S. M. Black, A. R. Sheldon: Neonatal mice lacking neuronal nitric oxide synthase are less vulnerable to hypoxic-ischemic injury. *Neurobiol Dis* 3:64-71 (1996)

75. Black SM, M. A. Bedolli, S. Martinez, J. D. Bristow, D. M. Ferriero, S. J. Soifer: Expression of neuronal nitric oxide synthase corresponds to regions of selective vulnerability to hypoxia-ischaemia in the developing rat brain. *Neurobiol Dis* 2:145-155 (1995)

76. Ishida A, W. H. Trescher, M. S. Lange, M. V. Johnston: Prolonged suppression of brain nitric oxide synthase activity by 7-nitroindazole protects against cerebral hypoxic-ischemic injury in neonatal rat. *Brain Dev* 23:349-354 (2001)

77. Peeters-Scholte C, J. Koster, E. van den Tweel, K. Blomgren, N. Hamers, C. Zhu, S. van Buul-Offers, H. Hagberg, F. van Bel, C. Heijnen, F. Groenendaal: Effects of selective nitric oxide synthase inhibition on IGF-1, caspases and cytokines in a newborn piglet model of perinatal hypoxia-ischaemia. *Dev Neurosci* 24:396-404 (2002)

78. Parikh NA, C. D. Katsetos, Q. M. Ashraf, S. H. Haider, A. Legido, M. Delivoria-Papadopoulos, O. P. Mishra: Hypoxia-induced caspase-3 activation and DNA fragmentation in cortical neurons of newborn piglets: role of nitric oxide. *Neurochem Res* 28:1351-1357 (2003)

79. Mishra OP, A. B. Zubrow, Q. M. Ashraf: Nitric oxide-mediated activation of extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK) during hypoxia in cerebral cortical nuclei of newborn piglets. *Neuroscience* 123:179-186 (2004)

80. Harris C, A. C. Maroney, E. M. Johnson: Identification of JNK-dependent and -independent components of cerebellar granule neuron apoptosis. *J Neurochem* 83:992-1001 (2002)

81. Wang X, C. Zhu, L. Qiu, H. Hagberg, M. Sandberg, K. Blomgren: Activation of ERK1/2 after neonatal rat cerebral hypoxia-ischaemia. *J Neurochem* 86:351-362 (2003)

82. Dammann O, A. Leviton: Role of the fetus in perinatal infection and neonatal brain damage. *Curr Opin Pediatr* 12:99-104 (2000)

83. Savman K, M. Blennow, K. Gustafson, E. Tarkowski, H. Hagberg: Cytokine response in cerebrospinal fluid after birth asphyxia. *Pediatr Res* 43:746-751 (1998)

84. Foster-Barber A, D. Ferriero: Neonatal encephalopathy in the term infant: neuroimaging and inflammatory cytokines. *Ment Retard Dev Disabil Res Rev* 8:20-24 (2002)

85. Wood PL: Microglia as a unique cellular target in the treatment of stroke: potential neurotoxic mediators produced by activated microglia. *Neurol Res* 17:242-248 (1995)

86. Tahraoui SL, S. Marret, C. Bodenant, P. Leroux, M. A. Dommergues, P. Evrard, P. Gressens: Central role of microglia in neonatal excitotoxic lesions of the murine periventricular white matter. *Brain Pathol* 11:56-71 (2001)

87. Dommergues MA, F. Plaisant, C. Verney, P. Gressens: Early microglial activation following neonatal excitotoxic brain damage in mice: a potential target for neuroprotection. *Neuroscience* 121:619-628 (2003)

88. Sheldon AR, C. Sedik, D. M. Ferriero: Strain-related brain injury in neonatal mice subjected to hypoxia-ischemia. *Brain Res* 810:114-122 (1998)

89. Yonekura I, N. Kawahara, H. Nakatomi, K. Furuya, T. Kirino: A model of global cerebral ischemia in C57 BL/6 mice. *J Cereb Blood Flow Metab* 24:151-158 (2004)

90. Banasiak KJ, Y. Xia, G. G. Haddad: Mechanisms underlying hypoxia-induced neuronal apoptosis. *Prog Neurobiol* 62:215-249 (2000)

91. Szolnoki Z, F. Somogyvári, A. Kondacs, M. Szabó, L. Fodor: Evaluation of the interactions of common genetic mutations in stroke subtypes. *J Neurol* 249:1391-1397 (2002)

92. Günther G, R. Junker, R. Sträter, R. Schobess, K. Kurnik, A. Kosch, U. Nowak-Göttl: Symptomatic ischemic stroke in full-term neonates : role of acquired and genetic prothrombotic risk factors. *Stroke* 31:2437-2441 (2000)

93. Nunez JL, M. McCarthy: Sex differences and hormonal effects in a model of preterm infant brain injury. *Ann N Y Acad Sci* 1008:281-4.:281-284 (2003)

94. Siegemund M, J. van Bommel, C. Ince: Assessment of regional tissue oxygenation. *Intensive Care Med* 25:1044–60 (1999)

95. Eken P, G. H. Jansen, F. Groenendaal, K. Rademaker, L. S. de Vries: Intracranial lesions in the fullterm infant with hypoxic ischaemic encephalopathy: ultrasound and autopsy correlation. *Neuropediatrics* 25:301–307 (1994)

96. Rutherford MA, J. M. Pennock, S. J. Counsell, E. Mercuri, F. M. Cowan, L. M. S. Dubowitz, D. A. Edwards: Abnormal magnetic resonance signal in the internal capsule predicts poor neurodevelopmental outcome in infants with hypoxic-ischemic encephalopathy. *Pediatrics* 102:323–328 (1998)

97. Martin E, J. A. Barkovich: Magnetic resonance imaging in perinatal asphyxia. *Arch Dis Child Fetal Neonatal Ed* 72: F62–70 (1995)

98. De Vries LS: Somatosensory evoked potentials in term neonates with postasphyxial encephalopathy. *Clin. Perinatol* 20:463–482 (1993)

99. Holmes G, J. Rowe, J. Hafford, R. Schmidt, M. Testa, A. Zimmerman: Prognostic value of the electroencephalogram in neonatal asphyxia. *Electroencephalogr Clin Neurophysiol* 53:60–72 (1982)

100. Toet MT, W. van der Meij, L. S. de Vries, A. C. van Huffelen: Comparison between simultaneously recorded amplitude integrated EEG cerebral function monitor and standard EEG in neonates. *Pediatrics* 109:772–779 (2002)

101. Jobsis FF: Noninvasive, infrared monitoring of cerebral and myocardial oxygen sufficiency and circulatory parameters. *Science* 198:1264–7 (1977)

102. Brazy JE, D. V. Lewis, M. H. Mitnick, F. F. J. van der Vliet: Non-invasive monitoring of cerebral oxygenation in preterm infants: preliminary observations. *Pediatrics* 75:217–25 (1985)

103. Watkin SL, S. A. Spencer, P. W. Dimmock, Y. A. Wickramasinghe, P. Rolfe: A comparison of pulse oximetry and near infrared spectroscopy (NIRS) in the detection of hypoxaemia occurring with pauses in nasal airflow in neonates. *J Clin Monit* 15:441–7 (1999)

104. Roche-Labarbe N, F. Wallois, E. Ponchel, G. Kongolo, R. Grebe: Coupled oxygenation oscillation measured by NIRS and intermittent cerebral activation on EEG in premature infants. *Neuroimage* 36:718-27 (2007)

105. Isobe K, T. Kusaka, Y. Fujikawa, K. Okubo, K. Nagano, S. Yasuda, M. Kondo, S. Itoh, K. Hirao, S. Onishi**:** Measurement of cerebral oxygenation in neonates after vaginal delivery and cesarean section using full spectrum near infrared spectroscopy *Comp. Biochem. Physiol.* A 132 133–8 (2002)

106. Naulaers G, G. Morren, S. Van Huffel, P. Casaer, H. Devlieger: Measurement of tissue oxygenation index during the first three days in premature born infants *Adv. Exp. Med. Biol.* 510:379–83 (2003)

107. Volpe JJ: Hypoxic-ischemic encephalopathy: clinical aspects. In: Neurology of the newborn. Eds: W.B. Saunders Company, Philadelphia (2001)

108. Tyszczuk L, J. Meek, C. Elwell, J. S. Wyatt: Cerebral blood flow is independent of mean arterial blood pressure in preterm infants undergoing intensive care. *Pediatrics* 102: 337–41 (1998)

109. Soul J, P. E. Hammer, M. Tsuji, P. J. Saul, H. Bassan, C. Limperopoulos, D. Disalvo, M. Moore, P. Akins, S. Ringer, J. J. Volpe, F. Trachtenberg, A. du Plessis: Fluctuating pressure-passivity is common in the cerebral circulation of sick premature infants. *Pediatr Res* 61:467-73 (2007)

110. Huang L, H. Ding, X. Hou, C. Zhou, G. Wang, F. Tian: Assessment of the hypoxic-ischemic encephalopathy in neonates using non-invasive near-infrared spectroscopy. *Physiol Meas* 25:749-61 (2004)

111. Meek JH, C. E. Elwell, D. C. McCormick, D. A. Edwards, J. P. Townsend, A. L. Stewart, J. S. Wyatt: Abnormal cerebral haemodynamics in perinatally asphyxiated neonates related to outcome. *Arch Dis Child Fetal Neonatal Ed* 81:110–5 (1999)

112. Zaramella P, E. Saraceni, F. Freato, E. Falcon, A. Suppiej, A. Milan, A. M. Laverda, L. Chiandetti: Can tissue oxygenation index (TOI) and cotside neurophysiological variables predict outcome in depressed/asphyxiated newborn infants? *Early Hum Dev* 83:483-9 (2007)

113. Vintzileos AM, S. Nioka, M. Lake, P. Li, Q. Luo, B. Chance**:** Transabdominal fetal pulse oximetry with nearinfrared spectroscopy. *Am J Obstet Gynecol* 192:129–33 (2005)

114. Bennet L, S. Rossenrode, M. I. Gunning, P. Gluckman, A. Gunn: The cardiovascular and cerebrovascular responses of the immature fetal sheep to acute umbilical cord occlusion. *J Physiol* 517:247–57 (1999)

115. Peebles DM, D. A. Edwards, J. S. Wyatt, A. P. Bishop, M. Cope, D. T. Delpy, O. E. Reynolds: Changes in human fetal cerebral haemoglobin concentration and oxygenation during labor measured by near-infrared spectroscopy. *Am J Obstet Gynecol* 166:624–8 (1992)

116. Faris F, M. Doyle, Y. Wickramasinghe, R. Houston, P. Rolfe, S. O'Brien: A non-invasive optical technique for

intrapartum fetal monitoring: preliminary clinical studies. *Med Eng Phys* 16: 287–91 (1994)

117. Nicklin SE, I. A. Hassan, Y. A. Wickramasinghe, S. A. Spencer: The light still shines, but not that brightly? The current status of perinatal near infrared spectroscopy. *Arch Dis Child Neonatal Ed* 88: F263–268 (2003)

118. Greisen G: Is near-infrared spectroscopy living up to its promises? *Semin Fetal Neonatal Med* 11:498-502 (2006)

119. Luisi S, P. Florio, F. M. Reis, F. Petraglia: Expression and secretion of activin A: possible physiological and clinical implications. *Eur J Endocrinol* 145:225-236 (2001)

120. Florio P, R. F. Abella, T. de la Torre, A. Giamberti, S. Luisi, G. Butera, A. Cazzaniga, A. Frigiola, F. Petraglia, D. Gazzolo: Perioperative activin A concentrations as a predictive marker of neurologic abnormalities in children after open heart surgery. *Clin Chem* 53:982-985 (2007)

121. Florio P, D. Gazzolo, S. Luisi, F. Petraglia: Activin A in brain injury. *Adv Clin Chem* 43:117-130 (2007)

122. Lai M, E. Sirimanne, C. E. Williams, P. D. Gluckman: Sequential patterns of inhibin subunit gene expression following hypoxic-ischemic injury in the rat brain. *Neuroscience* 70:1013-1024 (1996)

123. Wu DD, M. Lai, P. E. Hughes, E. Sirimanne, P. D. Gluckman, Chris E. Williams: Expression of the activin axis and neuronal rescue effects of recombinant activin A following hypoxic-ischemic brain injury in the infant rat. *Brain Res* 835:369-378 (1999)

124. Florio P, D. Gazzolo, S. Luisi, F. Petraglia: Activin A in brain injury. *Adv Clin Chem* 43:117-130 (2007)

125. Schubert D, H. Kimura, M. LaCorbiere, J. Vaughan, D. Karr, W. H. Fischer: Activin is a nerve cell survival molecule. *Nature* 344:868-870 (1990)

126. Kriegistein K, C. Suter-Crazzolara, W. H. Fischerl, K. Unsicker: TGF-beta superfamily members promote survival of midbrain dopaminergic neurons and protect them against MPP+ toxicity. *EMBO J* 14:736-742 (1995)

127. Iwahori Y, H. Saito, K. Torii, N. Nishiyama: Activin exerts a neurotrophic effect on cultured hippocampal neurons. *Brain Res* 760:52-58 (1997)

128. Hughes PE, T. Alexi, C. E. Williams, R. G. Clark, P. D. Gluckman: Administration of recombinant human Activin-A has powerful neurotrophic effects on select striatal phenotypes in the quinolinic acid lesion model of Huntington's disease. *Neuroscience* 92:197-209 (1999)

129. Rickmann M, J. R. Wolff: S100 protein expression in subpopulations of neurons of rat brain. *Neuroscience* 67:977-991 (1995)

130. Yang Q, A. Hamberger, H. Hyden, S. Wang, T. Stigbrand, K. G. Haglid: S-100 beta has a neuronal localisation in the rat hindbrain revealed by an antigen retrieval method. *Brain Res* 696:49-61 (1995)

131. Haimoto H, S. Hosoda, K. Kato: Differential distribution of immunoreactive S100-alpha and S100-beta proteins in normal nonnervous human tissues. *Lab Invest* 57:489-498 (1987)

132. Michetti F, E. Dell'Anna, G. Tiberio, D. Cocchia: Immunochemical and immunocytochemical study of S-100 protein in rat adipocytes. *Brain Res* 262:352-356 (1983)

133. Buyukuysal RL: Protein S100B release from rat brain slices during and after ischemia: comparison with lactate dehydrogenase leakage. *Neurochem Int* 47:580-588 (2005)

134. Gerlach R, G. Demel, H. G. Konig, U. Gross, J. H. Prehn, A. Raabe, V. Seifert, D. Kogel: Active secretion of S100B from astrocytes during metabolic stress. *Neuroscience* 141:1697-1701 (2006)

135. Abraha HD, R. J. Butterworth, P. M. Bath, W. S. Wassif, J. Garthwaite, R. A. Sherwood: Serum S-100 protein, relationship to clinical outcome in acute stroke. *Ann Clin Biochem* 34:546-550 (1997)

136. Kanner AA, N. Marchi, V. Fazio, M. R. Mayberg, M. T. Koltz, V. Siomin, G. H. J. Stevens, T. Masaryk, B. Ayumar, M. A. Vogelbaum, G. H. Barnett, D. Janigro: Serum S100beta: a noninvasive marker of blood-brain barrier function and brain lesions. *Cancer* 97:2806-2813 (2003)

137. Hu J, F. Castets, J. L. Guevara, L. J. Van Eldik: S100 beta stimulates inducible nitric oxide synthase activity and mRNA levels in rat cortical astrocytes. *J Biol Chem* 271:2543-2547 (1996)

138. Aurell A, L. E. Rosengren, B. Karlsson, J. E. Olsson, V. Zbornikova, K. G. Haglid: Determination of S-100 and glial fibrillary acidic protein concentrations in cerebrospinal fluid after brain infarction. *Stroke* 22:1254-1258 (1991)

139. Wakita H, H. Tomimoto, I. Akiguchi, J. Kimura: Glial activation and white matter changes in the rat brain induced by chronic cerebral hypoperfusion: an immunohistochemical study. *Acta Neuropathol* 87:484-492 (1994)

140. Tomimoto H, M. Ihara, H. Wakita, R. Ohtani R, J. X. Lin, I. Akiguchi, M. Kinoshita, H. Shibasaki: Chronic cerebral hypoperfusion J Cereb Blood Flow Metab induces white matter lesions and loss of oligodendroglia with DNA fragmentation in the rat. *Acta Neuropathol* 106:527-534 (2003)

141. Ueno M, H. Tomimoto, I. Akiguchi, H. Wakita, H. Sakamoto: Blood-brain barrier disruption in white matter

lesions in a rat model of chronic cerebral hypoperfusion. *J Cereb Blood Flow Metab* 22:97-104 (2002)

142. Nawashiro H, M. Brenner, S. Fukui, K. Shima, J. M. Hallenbeck: High susceptibility to cerebral ischemia in GFAP-null mice. *J Cereb Blood Flow Metab* 20:1040-1044 (2000)

143. Guo L, A. Sawkar, M. Zasadzki, D. M. Watterson, L. J. Van Eldik: Activation of glial cultures from different rat brain regions by neuroinflammatory stimuli and downregulation of the activation by a new class of small molecule ligands. *Neurobiol Aging* 22:975-981 (2001)

144. Murphy S: Production of nitric oxide by glial cells: regulation and potential roles in the CNS. *Glia* 29:1-13 (2000)

145. Lam AGM, T. Koppal, K. T. Akama, L. Guo, J. M. Craft, B. Samy, J. P. Schavocky, D. M. Watterson, L. J. Van Eldik: Mechanism of glial activation by S100B: involvement of the transcription factor NFkappaB. *Neurobiol Aging* 22:765-772 (2001)

146. Sharp FR, A. Lu, Y. Tang, D. Millhorn: Multiple molecular penumbras after focal cerebral ischemia. *J Cereb Blood Flow Metab* 20:1011-1032 (2000)

147. Asano T, T. Mori, T. Shimoda, R. Shinagawa, S. Satoh, N. Yada, S. Katsumata, M. S, Y. Kagamiishi, N. Tateishi: Arundic acid (ONO-2506) ameliorates delayed ischemic brain damage by preventing astrocytic overproduction of S100B. *Curr Drug Targets CNS Neurol Disord* 4:127-142 (2005)

148. Matsui T, T. Mori, N. Tateishi, Y. Kagamiishi, S. Satoh, N. Katsube, E. Morikawa, T. Morimoto, F. Ikuta, T. Asano: Astrocytic activation and delayed infarct expansion after permanent focal ischemia in rats. Part I: enhanced astrocytic synthesis of s-100beta in the periinfarct area precedes delayed infarct expansion. *J Cereb Blood Flow Metab* 22:711-722 (2002)

149. Ohtani R, H. Tomimoto, H. Wakita, H. Kitaguchi, K. Nakaji, R. Takahashi: Expression of S100 protein and protective effect of arundic acid on the rat brain in chronic cerebral hypoperfusion. *Brain Res* 1135:195-200 (2007)

150. Hinson JP, S. Kapas, D. M. Smith: Adrenomedullin, a multifunctional regulatory peptide. *Endocr Rev* 21:138-167 (2000)

151. Kitamura K, K. Kangawa, M. Kawamoto, Y. Ichiki, S. Nakamura, H. Matsuo, T. Eto: Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun* 192:553-560 (1993)

152. Allen MA, A. V. Ferguson: In vitro recordings from area postrema neurons demonstrate responsiveness to adrenomedullin. *Am J Physiol* 270:R920-R925 (1996)

153. Kato H, M. Shichiri, F. Marumo, Y. Hirata: Adrenomedullin as an autocrine/paracrine apoptosis survival factor for rat endothelial cells. *Endocrinology* 138:2615-2620 (1997)

154. Oehler MK, C. Norbury, S. Hague, C. M. Rees, R. Bicknell: Adrenomedullin inhibits hypoxic cell death by upregulation of Bcl-2 in endometrial cancer cells: a possible promotion mechanism for tumour growth. *Oncogene* 20:2937-2945 (2001)

155. Wang X, T. L. Yue, F. C. Barone, R. F. White, R. K. Clark, R. N. Willette, A. C. Sulpizio, N. V. Aiyar, R. R. Ruffolo, G. Z. Feuerstein: Discovery of adrenomedullin in rat ischemic cortex and evidence for its role in exacerbating focal brain ischemic damage. *Proc Natl Acad Sci U S A* 92:11480-11484 (1995)

156. Nagata D, Y. Hirata, E. Suzuki, M. Kakoki, H. Hayakawa, A. Goto, T. Ishimitsu, N. Minamino, Y. Ono, K. Kangawa, H. Matsuo, M. Omata: Hypoxia-induced adrenomedullin production in the kidney. *Kidney Int* 55:1259-1267 (1999)

157. Ueda S, K. Nishio, N. Minamino, A. Kubo, Y. Akai, K. Kangawa, H. Matsuo, Y. Fujimura, A. Yoshioka, K. Masui, N. Doi, Y. Murao, S. Miyamoto: Increased plasma levels of adrenomedullin in patients with systemic inflammatory response syndrome. *Am J Respir Crit Care Med* 160:132-136 (1999)

158. Caron KM, O. Smithies: Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional Adrenomedullin gene. *Proc Natl Acad Sci U S A* 98:615-619 (2001)

159. Shimosawa T, Y. Shibagaki, K. Ishibashi, K. Kitamura, K. Kangawa, S. Kato, K. Ando, T. Fujita: Adrenomedullin, an endogenous peptide, counteracts cardiovascular damage. *Circulation* 105:106-111 (2002)

160. Shindo T, Y. Kurihara, H. Nishimatsu, N. Moriyama, M. Kakoki, Y. Wang, Y. Imai, A. Ebihara, T. Kuwaki, K. H. Ju, N. Minamino, K. Kangawa, T. Ishikawa, M. Fukuda, Y. Akimoto, H. Kawakami, T. Imai, H. Morita, Y. Yazaki, R. Nagai, Y. Hirata, H. Kurihara: Vascular abnormalities and elevated blood pressure in mice lacking adrenomedullin gene. *Circulation* 104:1964-1971 (2001)

161. Ueta Y, K. Kitamura, T. Isse, I. Shibuya, N. Kabashima, S. Yamamoto, K. Kangawa, H. Matsuo, T. Eto, H. Yamashita: Adrenomedullin-immunoreactive neurons in the paraventricular and supraoptic nuclei of the rat. Neurosci Lett 202:37-40 (1995)

162. Sone M, K. Takahashi, F. Satoh, O. Murakami, K. Totsune, M. Ohneda, H. Sasano, H. Ito, T. Mouri: Immunocytochemical localization of adrenomedullin-like immunoreactivity in the human hypothalamus and the adrenal gland. *Neurosci Lett* 203:207-210 (1996)

163. Garayoa M, A. Martínez, S. Lee, R. Pío, W. G. An, L. Neckers, J. Trepel, L. M. Montuenga, H. Ryan, R. Johnson, M. Gassmann, F. Cuttitta: Hypoxia-inducible factor-1 (HIF-1) up-regulates adrenomedullin expression in human tumor cell lines during oxygen deprivation: a possible promotion mechanism of carcinogenesis. *Mol Endocrinol* 14:848-862 (2000)

164. Serrano J, D. Alonso, J. M. Encinas, J. C. Lopez, A. P. Fernandez, S. Castro-Blanco, P. Fernandez-Vizarra, A. Richart, M. L. Bentura, M. Santacana, L. O. Uttenthal, F. Cuttitta, J. Rodrigo, A. Martinez: Adrenomedullin expression is up-regulated by ischemia-reperfusion in the cerebral cortex of the adult rat. *Neuroscience* 109:717-731 (2002)

165. Encinas JM, J. Serrano, D. Alonso, A. P. Fernández, J. Rodrigo: Adrenomedullin over-expression in the caudate-putamen of the adult rat brain after ischaemia-reperfusion injury. *Neurosci Lett* 329:197-200 (2002)

166. Bernaudin M, Y. Tang, M. Reilly, E. Petit, F. R. Sharp: Brain genomic response following hypoxia and re-oxygenation in the neonatal rat. Identification of genes that might contribute to hypoxia-induced ischemic tolerance. *J Biol Chem* 277:39728-39738 (2002)

167. Serrano J, D. Alonso, A. P. Fernandez, J. M. Encinas, J. C. Lopez, S. Castro-Blanco, P. Fernandez-Vizarra, A. Richart, M. Santacana, L. O. Uttenthal, M. L. Bentura, R. Martinez-Murillo, A. Martinez, F. Cuttitta, J. Rodrigo: Adrenomedullin in the central nervous system. *Microsc Res Tech* 57:76-90 (2002)

168. Dogan A, Y. Suzuki, N. Koketsu, K. Osuka, K. Saito, M. Takayasu, M. Shibuya, J. Yoshida: Intravenous infusion of adrenomedullin and increase in regional cerebral blood flow and prevention of ischemic brain injury after middle cerebral artery occlusion in rats. *J Cereb Blood Flow Metab* 17:19-25 (1997)

169. Watanabe K, M. Takayasu, A. Noda, M. Hara, T. Takagi, Y. Suzuki, J. Yoshia: Adrenomedullin reduces ischemic brain injury after transient middle cerebral artery occlusion in rats. *Acta Neurochir (Wien)* 143:1157-1161 (2001)

170. Xia CF, H. Yin, C. V. Borlongan, J. Chao, L. Chao: Adrenomedullin gene delivery protects against cerebral ischemic injury by promoting astrocyte migration and survival. Hum Gene Ther 15:1243-1254 (2004)

171. Miyashita K, H. Itoh, H. Arai, T. Suganami, N. Sawada, Y. Fukunaga, M. Sone, K. Yamahara, T. Yurugi-Kobayashi, K. Park, N. Oyamada, N. Sawada, D. Taura, H. Tsujimoto, T. H. Chao, N. Tamura, M. Mukoyama, K. Nakao: The neuroprotective and vasculo-neuro-regenerative roles of adrenomedullin in ischemic brain and its therapeutic potential. Endocrinology 147:1642-1653 (2006)

172. Pahlman S, T. Esscher, P. Bergvall, L. Odelstad: Purification and characterization of human neuron-specific enolase: radioimmunoassay development. *Tumour Biol* 5:127-139 (1984)

173. Iwanaga T, Y. Takahashi, T. Fujita: Immunohistochemistry of neuron-specific and glia-specific proteins. *Arch Histol Cytol* 52:13-24 (1989)

174. Barone FC, R. K. Clarka, W. J. Price, R. F. White, G. Z. Feuerstein, B. L. Storer, E. H. Ohlstein: Neuron-specific enolase increases in cerebral and systemic circulation following focal ischemia. *Brain Res* 623:77-82 (1993)

175. Xanthosa T, K. A. Ekmektzoglou, L. Papadimitriou: Biochemical markers (NSE, S-100, IL-8) as predictors of neurological outcome in patients after cardiac arrest and return of spontaneous circulation. *Resuscitation* 75:219-228 (2007)

176. Lamers KJB, P. Vos, M. M. Verbeek, F. Rosmalen , W. J.A. van Geel, B. G.M. van Engelen: Protein S-100B, neuron-specific enolase (NSE), myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP) in cerebrospinal fluid (CSF) and blood of neurological patients. *Brain Res Bull* 61:261-264 (2003)

177. Johnsson P, S. Blomquist, C. Lührs, G. Malmkvist, C. Alling, J. O. Solem, E. Ståhl: Neuron-specific enolase increases in plasma during and immediately after extracorporeal circulation. *Ann Thorac Surg* 69:750-754 (2000)

178. Ramont L, H. Thoannes, A. Volondat, F. Chastang, M. C. Millet, F. X. Maquart: Effects of hemolysis and storage condition on neuron-specific enolase (NSE) in cerebrospinal fluid and serum: implications in clinical practice. *Clin Chem Lab Med* 43:1215-1217 (2005)

179. Takei N, J. Kondo, K. Nagaike, K. Ohsawa, K. Kato, S. Kohsaka: Neuronal survival factor from bovine brain is identical to neuron-specific enolase. *J Neurochem* 57:1178-1184 (1991)

180. Hattori T, K. Ohsawa, Y. Mizuno, K. Kato, S. Kohsaka: Synthetic peptide corresponding to 30 amino acids of the C-terminal of neuron-specific enolase promotes survival of neocortical neurons in culture. *Biochem Biophys Res Commun* 202:25-30 (1994)

181. Hattori T, N. Takei, Y. Mizuno, K. Kato, S. Kohsaka: Neurotrophic and neuroprotective effects of neuron-specific enolase on cultured neurons from embryonic rat brain. *Neurosci Res* 21:191-198 (1995)

182. Nedergaard M, U. Dirnagl: Role of glial cells in cerebral ischemia. *Glia* 50:281-286 (2005)

183. Panickar KS, M. D. Norenberg: Astrocytes in cerebral ischemic injury: morphological and general considerations. *Glia* 50:287-298 (2005)

184. Plum F: What causes infarction in ischemic brain? The Robert Wartenberg Lecture. *Neurology* 33:222-233 (1983)

185. Garcia JH, Y. Yoshida, H. Chen, Y. Li, Z. G. Zhang, J. Lian, S. Chen, M. Chopp: Progression from ischemic injury to infarct following middle cerebral artery occlusion in the rat. *Am J Pathol* 142:623-635 (1993)

186. Martin LJ, A. M. Brambrink, C. Lehmann, C. Portera-Cailliau, R. Koehler, J. Rothstein, R. J. Traystman: Hypoxia-ischemia causes abnormalities in glutamate transporters and death of astroglia and neurons in newborn striatum. *Ann Neurol* 42:335-348 (1997)

187. Lukaszevicz AC, N. Sampaïo, C. Guégan, A. Benchoua, C. Couriaud, E. Chevalier, B. Sola, P. Lacombe, B. Onténiente: High sensitivity of protoplasmic cortical astroglia to focal ischemia. *J Cereb Blood Flow Metab* 22:289-298 (2002)

188. Missler U, M. Wiesmann, G. Wittmann, O. Magerkurth, H. Hagenström: Measurement of glial fibrillary acidic protein in human blood: analytical method and preliminary clinical results. *Clin Chem* 45:138-141 (1999)

189. Pelinka LE, A. Kroepfl, M. Leixnering, W. Buchinger, A. Raabe, H. Redl: GFAP versus S100B in serum after traumatic brain injury: relationship to brain damage and outcome. *J Neurotrauma* 21:1553-1561 (2004)

190. Herrmann M, P. Vos, M. T. Wunderlich, C. H. M. M. de Bruijn, K. J. B. Lamers: Release of glial tissue-specific proteins after acute stroke: A comparative analysis of serum concentrations of protein S-100B and glial fibrillary acidic protein. *Stroke* 31:2670-2677 (2000)

191. Yasuda Y, N. Tateishi, T. Shimoda, S. Satoh, E. Ogitani, S. Fujita: Relationship between S100beta and GFAP expression in astrocytes during infarction and glial scar formation after mild transient ischemia. *Brain Res* 1021:20-31 (2004)

192. Foerch C, I. Curdt, B. Yan, F. Dvorak, M. Hermans, J. Berkefeld, A. Raabe, T. Neumann-Haefelin, H. Steinmetz, M. Sitzer: Serum glial fibrillary acidic protein as a biomarker for intracerebral haemorrhage in patients with acute stroke. *J Neurol Neurosurg Psychiatry* 77:181-184 (2006)

193. Vos PE, M. van Gils, T. Beems, C. Zimmerman, M. M Verbeek: Increased GFAP and S100beta but not NSE serum levels after subarachnoid haemorrhage are associated with clinical severity. *Eur J Neurol* 13:632-638 (2006)

194. Wunderlich MT, C. W. Wallesch, M. Goertler: Release of glial fibrillary acidic protein is related to the neurovascular status in acute ischemic stroke. *Eur J Neurol* 13:1118-1123 (2006)

195. Rosengren LE, C. Wikkelso, L. Hagberg: A sensitive ELISA for glial fibrillary acidic protein: application in CSF of adults. *J Neurosci Methods* 51:197-204 (1994)

196. Verbeek MM, D. De Jong, B. P.H. Kremer: Brain-specific proteins in cerebrospinal fluid for the diagnosis of neurodegenerative diseases. *Ann Clin Biochem* 40:25-40 (2003)

197. Norgren N, P. Sundstrom, A. Svenningsson, L. Rosengren, T. Stigbrand, M. Gunnarsson: Neurofilament and glial fibrillary acidic protein in multiple sclerosis. *Neurology* 63:1586-1590 (2004)

198. Blennow M, H. Hagberg, L. Rosengren: Glial fibrillary acidic protein in the cerebrospinal fluid: a possible indicator of prognosis in full-term asphyxiated newborn infants? *Pediatr Res* 37:260-264 (1995)

199. Blennow M, L. Rosengren, S. Jonsson, H. Forssberg, M. Katz-Salamon, H. Hagberg, U. Hesser, H. Lagercrantz: Glial fibrillary acidic protein is increased in the cerebrospinal fluid of preterm infants with abnormal neurological findings. *Acta Paediatr* 85:485-489 (1996)

200. Florio P, S. Perrone, S. Luisi, P. Vezzosi, M. Longini, B. Marzocchi, F. Petraglia, G. Buonocore: Increased plasma concentrations of activin a predict intraventricular hemorrhage in preterm newborns. *Clin Chem* 52:1516-1521 (2006)

201. Florio P, S. Luisi, M. Bruschettini, D. Grutzfeld, A. Dobrzanska, P. Bruschettini, F. Petraglia, D. Gazzolo: Cerebrospinal fluid activin a measurement in asphyxiated full-term newborns predicts hypoxic ischemic encephalopathy. *Clin Chem* 50:2386-2389 (2004)

202. Florio P, S. Luisi, B. Moataza, M. Torricelli, I. Iman, M. Hala, A. Hanna, F. Petraglia, D. Gazzolo: High urinary concentrations of activin A in asphyxiated full-term newborns with moderate or severe hypoxic ischemic encephalopathy. *Clin Chem* 53:520-522 (2007)

203. Michetti F, D. Gazzolo: S100B testing in pregnancy. *Clin Chim Acta* 335:1-7 (2003)

204. Michetti F, A. Massaro, M. Murazio: The nervous system-specific S-100 antigen in cerebrospinal fluid of multiple sclerosis patients. *Neurosci Lett* 11:171-175 (1979)

205. Michetti F, A. Massaro, G. Russo, G. Rigon: The S-100 antigen in cerebrospinal fluid as a possible index of cell injury in the nervous system. *J Neurol Sci* 44:259-263 (1980)

206. Whitelaw A, L. Rosengren, M. Blennow: Brain specific proteins in posthaemorrhagic ventricular dilatation. *Arch Dis Child Fetal Neonatal Ed* 84:F90-F91 (2001)

207. Blennow M, K. Savman, P. Ilves, M. Thoresen, L. Rosengren: Brain-specific proteins in the cerebrospinal fluid of severely asphyxiated newborn infants. *Acta Paediatr* 90:1171-1175 (2001)

208. Sellman M, T. Ivert, G. Ronquist, K. Caesarini, L. Persson, B. K. Semb: Central nervous system damage during cardiac surgery assessed by 3 different biochemical

markers in cerebrospinal fluid. *Scand J Thorac Cardiovasc Surg* 26:39-45 (1992)

209. Persson L, H. G. Hardemark, J. Gustafsson, G. Rundstrom, I. Mendel-Hartvig, T. Esscher, S. Pahlman: S-100 protein and neuron-specific enolase in cerebrospinal fluid and serum: markers of cell damage in human central nervous system. *Stroke* 18:911-918 (1987)

210. Gazzolo D, P. Vinesi, M. Bartocci, M. C. Geloso, W. Bonacci, G. Serra, K. G. Haglid, F. Michetti: Elevated S100 blood level as an early indicator of intraventricular hemorrhage in preterm infants. Correlation with cerebral Doppler velocimetry. *J Neurol Sci* 170:32-35 (1999)

211. Gazzolo D, R. di Iorio, E. Marinoni, P. Masetti, G. Serra, L. Giovannini, F. Michetti: S100B protein is increased in asphyxiated term infants developing intraventricular hemorrhage. *Crit Care Med* 30:1356-1360 (2002)

212. Nagdyman N, W. Komen, H. K. Ko, C. Muller, M. Obladen: Early biochemical indicators of hypoxic-ischemic encephalopathy after birth asphyxia. *Pediatr Res* 49:502-506 (2001)

213. Gazzolo D, P. Masetti, M. Meli, D. Grutzfeld, F. Michetti: Elevated S100B protein as an early indicator of intracranial haemorrhage in infants subjected to extracorporeal membrane oxygenation. *Acta Paediatr* 91:218-221 (2002)

214. Golej J, G. Trittenwein: Early detection of neurologic injury and issues of rehabilitation after pediatric cardiac extracorporeal membrane oxygenation. *Artif Organs* 23:1020-1025 (1999)

215. Gazzolo D, E. Marinoni, R. di Iorio, M. Lituania, P. Bruschettini, F. Michetti: Circulating S100beta protein is increased in intrauterine growth-retarded fetuses. *Pediatr Res* 51:215-219 (2002)

216. Gazzolo D, E. Marinoni, R. di Iorio, M. Lituania, M. Marras, M. Bruschettini, P. Bruschettini, R. Frulio, F. Michetti, F. Petraglia, P. Florio: High maternal blood S100B concentrations in pregnancies complicated by intrauterine growth restriction and intraventricular hemorrhage. *Clin Chem* 52:819-826 (2006)

217. Gazzolo D, M. Bruschettini, M. Lituania, G. Serra, E. Gandullia, F. Michetti: S100b protein concentrations in urine are correlated with gestational age in healthy preterm and term newborns. *Clin Chem* 47:1132-1133 (2001)

218. Gazzolo D, M. Bruschettini, M. Lituania, G. Serra, W. Bonacci, F. Michetti: Increased urinary S100B protein as an early indicator of intraventricular hemorrhage in preterm infants: correlation with the grade of hemorrhage. *Clin Chem* 47:1836-1838 (2001)

219. Gazzolo D, E. Marinoni, R. di Iorio, M. Bruschettini, M. Kornacka, M. Lituania, U. Majewska, G. Serra, F. Michetti: Measurement of urinary S100B protein concentrations for the early identification of brain damage in asphyxiated full-term infants. Arch Pediatr Adolesc Med 157:1163-1168 (2003)

220. Boldt T, P. Luukkainen, F. Fyhrquist, M. Pohjavuori, S. Andersson: Birth stress increases adrenomedullin in the newborn. *Acta Paediatr* 87:93-94 (1998)

221. de Vroomen M, Y. Takahashi, V. Gournay, C. Roman, A. M. Rudolph, M. A. Heymann: Adrenomedullin increases pulmonary blood flow in fetal sheep. *Pediatr Res* 41:493-497 (1997)

222. Yamashiro C, K. Hayashi, T. Yanagihara, T. Hata: Plasma adrenomedullin levels in pregnancies with appropriate for gestational age and small for gestational age infants. *J Perinat Med* 29:513-518 (2001)

223. Akturk A, E. E. Onal, Y. Atalay, M. Yurekli, D. Erbas, N. Okumus, C. Turkyilmaz, S. Unal, E. Ergenekon, E. Koc, O. Himmetoglu: Maternal and umbilical venous adrenomedullin and nitric oxide levels in intrauterine growth restriction. *J Matern Fetal Neonatal Med* 20:521-525 (2007)

224. Trollmann R, E. Schoof, E. Beinder, D. Wenzel, W. Rascher, J. Dotsch: Adrenomedullin gene expression in human placental tIssue and leukocytes: a potential marker of severe tIssue hypoxia in neonates with birth asphyxia. *Eur J Endocrinol* 147:711-716 (2002)

225. di Iorio R, E. Marinoni, M. Lituania, G. Serra, L. Claudio, E. V. Cosmi, D. Gazzolo: Adrenomedullin increases in term asphyxiated newborns developing intraventricular hemorrhage. *Clin Biochem* 37:1112-1116 (2004)

226. de Herder WW: Biochemistry of neuroendocrine tumours. *Best Pract Res Clin Endocrinol Metab* 21:33-41(2007)

227. Thornberg E, K. Thiringer, H. Hagberg, I. Kjellmer: Neuron specific enolase in asphyxiated newborns: association with encephalopathy and cerebral function monitor trace. *Arch Dis Child Fetal Neonatal Ed* 72:F39-F42 (1995)

228. Garcia-Alix A, F. Cabanas, A. Pellicer, A. Hernanz, T. A. Stinis, J. Quero: Neuron-specific enolase and myelin basic protein: relationship of cerebrospinal fluid concentrations to the neurologic condition of asphyxiated full-term infants. *Pediatrics* 93:234-240 (1994)

229. Ezgu FS, Y. Atalay, K. Gucuyener, S. Tunc, E. Koc, E. Ergenekon, U. Tiras: Neuron-specific enolase levels and neuroimaging in asphyxiated term newborns. *J Child Neurol* 17:824-829 (2002)

230. Celtik C, B. Acunas, N. Oner, O. Pala: Neuron-specific enolase as a marker of the severity and outcome of hypoxic ischemic encephalopathy. *Brain Dev* 26:398-402 (2004)

231. Nagdyman N, I. Grimmer, T. Scholz, C. Muller, M. Obladen: Predictive value of brain-specific proteins in serum for neurodevelopmental outcome after birth asphyxia. *Pediatr Res* 54:270-275 (2003)

232. Picciano MF: Human milk: nutritional aspects of a dynamic food. *Biol Neonate* 74:84-93 (1998)

233. Lönnerdal B: Biochemistry and physiological function of human milk proteins. *Am J Clin Nutr* 42:1299-317 (1985)

234. Grosvenor CE, M. F. Picciano, C. R. Baumrucker: Hormones and growth factors in milk. *Endocr Rev* 14(6):710-28. (1993)

235. Neil G: Nutrition and cognitive function. *Brain Dev* 19:165-70 (1997)

236. Dangour A, R. Uauy: N-3 long-chain polyunsaturated fatty acids for optimal function during brain development and ageing. *Asia Pac J Clin Nutr* 17:185-8 (2008)

237. Belkind-Gerson J, A. Carreón-Rodríguez, C. O. Contreras-Ochoa, S. Estrada-Mondaca, M.S. Parra-Cabrera: Fatty acids and neurodevelopment. *J Pediatr Gastroenterol Nutr* 47:S7-S9 (2008)

238. Wurtman RJ: Synapse formation and cognitive brain development: effect of docosahexaenoic acid and other dietary constituents. *Metabolism* 57:6-10 (2008)

239. Amin SB, K. S. Merle, M. S. Orlando, L. E. Dalzell, R. Guillet: Brainstem maturation in premature infants as a function of enteral feeding type. *Pediatrics* 106:318-22 (2000)

240. Wang B, P. McVeagh, P. Petocz, J. Brand-Miller: Brain ganglioside and glycoprotein sialic acid in breastfed compared with formula-fed infants. *Am J Clin Nutr* 78:1024-9 (2003)

241. Schanler RJ, S. A. Atkinson: Effects of nutrients in human milk on the recipient premature infant. *J Mammary Gland Biol Neoplasia* 4:297-307 (1999)

242. Gazzolo D, G. Monego, V. Corvino, M. Bruschettini, P. Bruschettini, G. Zelano, F. Michetti: Human milk contains S100B protein. *Biochim Biophys Acta* 20:1619:209-12 (2003)

243. Michetti F, D. Gazzolo: S100B protein in biological fluids: a tool for perinatal medicine. *Clin Chem* 4:2097-104 (2002)

244. Gazzolo D, G. H. Visser, M. Lituania, R. Sarli, M. Bruschettini, F. Michetti, P. Bruschettini: S100B protein cord blood levels and development of fetal behavioral states: a study in normal and small-for-dates fetuses. *J Matern Fetal Neonatal Med* 11:378-84(2002)

245. Gazzolo D, M. Bruschettini, V. Corvino, R. Oliva, R. Sarli, M. Lituania, P. Bruschettini, F. Michetti**:** S100B protein concentrations in amniotic fluid correlate with gestational age and with cerebral ultrasound scanning results in healthy fetuses. *Clin Chem* 47:954-6 (2001)

246. Mather IH, T. W. Keenan: Origin and secretion of milk lipids. *J. Mammary Gland Biol. Neoplasia* 3:259– 273 (1998)

247. Gazzolo D, M. Bruschettini, M. Lituania, G. Serra, P. Santini, F. Michetti: Levels of S100B protein are higher in mature human milk than in colostrum and milk-formulae milks. *Clin Nutr* 23:23-6 (2004)

248. Nigro F, L. Gagliardi, S. Ciotti, F. Galvano, A. Pietri, G. L. Tina, D. Cavallaio, L. La Fauci, L. Iacopino, M. Bognanno, G. Li Volti, A. Scacco, F. Michetti, D. Gazzolo: S100B Protein concentration in milk-formulas for preterm and term infants. Correlation with industrial preparation procedures. *Mol Nutr Food Res* 52:609-13 (2008)

249. Pio R, A. Martínez, T. H. Elsasser, F. Cuttitta: Presence of immunoreactive adrenomedullin in human and bovine milk. *Peptides.* 21:1859-63 (2000)

250. Jahnke GD, M. J. Miller, A. Martínez, L. Montuenga, F. Cuttitta: Adrenomedullin expression in the mouse mammary gland: evidence for the mature form in milk. *J Mol Endocrinol* 19:279-89 (1997)

251. Ohta N, H. Tsukahara, Y. Ohshima, M. Nishii, Y. Ogawa, K. Sekine, K. Kasuga, M. Mayumi: Nitric oxide metabolites and adrenomedullin in human breast milk. *Early Hum Dev* 78:61-5 (2004)

252. Cekmen MB, A. Balat, O. Balat, F. Aksoy, M. Yurekli, A. B. Erbagci, S. Sahinoz: Decreased adrenomedullin and total nitrite levels in breast milk of preeclamptic women. *Clin Biochem* 37:146-8 (2004)

253. Luisi S, G. Calonaci, P. Florio, I. Lombardi, C. De Felice, F. Bagnoli, F. Petraglia: Identification of activin A and follistatin in human milk. *Growth Factors* 20:147-50 (2002)

254. Robinson GW, L. Hennighausen**:** Inhibins and activins regulate mammary epithelial cell differentiation through mesenchymal-epithelial interactions. *Development* 124:2701-8 (1997)

255. Reis FM, S. Luisi, M. M. Carneiro, L. Cobellis, M. Federico, A. F. Camargos, P. Felice: Activin, inhibin and the human breast. *Mol Cell Endocrinol* 225:77-82 (2004)

256. Welsch U, U. Pia, E. Hofter, F. Cuttitta, A. Martinez: Adrenomedullin in mammalian and human skin glands including the mammary gland. *Acta Histochem* 104:65-72 (2002)

Abbreviations: H-I: hypoxic-ischemic, CNS: central nervous system, AM: adrenomedullin, NSE: neuronal

specific enolase, GFAP: glial fibrillary acid protein, HIE: hypoxic ischemic encephalopathy, IVH: intraventricular hemorrhage, CBF: cerebral blood flow, CBV: cerebral blood volume, NIRS: near-infrared spectrometry, WM: white matter.

Key Words: Hypoxia-Ischemia, Brain Damage, Adrenomedullin, Activin A, S100B, NSE, GFAP, Review

Send correspondence to: Diego Gazzolo, Department of Maternal Fetal and Neonatal Health, G. Garibaldi Hospital, Via Palermo 636, I-95100 Catania, Italy, Tel: 39-95-7595215, Dax: 39-95-7595208, E-mail: dgazzolo@hotmail.com

http://www.bioscience.org/current/vol2S.htm