



# Biomarkers of Oxidative Stress in Neonatal Hypoxic-Ischemic Encephalopathy 11

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R. Rajendram et al. (eds.), *Biomarkers in Trauma, Injury and Critical Care*, Biomarkers  
in Disease: Methods, Discoveries and Applications,  
[https://doi.org/10.1007/978-3-031-07395-3\\_12](https://doi.org/10.1007/978-3-031-07395-3_12)

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## Abstract

Neonatal hypoxic-ischemic encephalopathy (HIE) results from the disruption of cerebral oxygen delivery and is a major cause of disability worldwide. The oxidative burst triggered by hypoxia-ischemia-reperfusion, glutamate excitotoxicity, and mitochondrial dysfunction plays a key role in the development of brain injury. Hence, multiple biomarkers of oxidative stress have been explored in asphyxiated infants and are reviewed in this chapter. While lipid and protein oxidation biomarkers, nonprotein-bound iron, and uric acid have long been studied, bringing encouraging predictive data on HIE severity and outcome, other biomarkers have been explored more recently and require further investigations. Oxidative biomarkers could be useful to identify infants at higher risk of moderate to severe HIE that would benefit from neuroprotective treatments. To date, however, they are not part of routine neonatal practice, mainly due to the high costs and complexity of the spectroscopic techniques required for their assessment.

## Keywords

Hypoxic-ischemic encephalopathy · Perinatal asphyxia · Neonate · Infant · Oxidative stress · Free radicals · Biomarkers · Lipid peroxidation · DNA oxidation · Protein oxidation · Nitric oxide · Acid uric · Nonprotein bound iron · Bilirubin · Uric acid

## Abbreviations

4-HNE	4-hydroxynonenal
8-OHdG	8-hydroxydeoxyguanosine
aEEG	Amplitude-integrated electroencephalography
AOPP	Advanced oxidation protein products
ATP	Adenosine triphosphate
CAT	Catalase
CNS	Central nervous system
Cr	Creatinine
CSF	Cerebrospinal fluid
EEG	Electroencephalography
ETC	Electron transport chain
Gal-3	Galectin-3
GC-MS/MS	Gas chromatography coupled to tandem mass spectrometry
GP	Glutathione peroxidase
HIE	Hypoxic-ischemic encephalopathy
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
MDA	Malondialdehyde
MRI	Magnetic resonance
MS	Mass spectrometry
NAD	Nicotinamide adenine dinucleotide

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NADPH	Nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NPBI	Nonprotein bound iron
O <sub>2</sub>	Molecular oxygen
PC	Protein carbonyls
PPPs	Prostaglandin-like peroxidation products
PUFAs	Polyunsaturated fatty acids
Quin	Quinolinic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
sTB	Serum total bilirubin
TH	Therapeutic hypothermia
UA	Uric acid
XD	Xanthine dehydrogenase
XO	Xanthine oxidase

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## Introduction

### Oxidative Stress

Molecular oxygen (O<sub>2</sub>) is key to guarantee aerobic metabolism via the mitochondrial oxidative phosphorylation. The electron transport chain in the inner mitochondrial membrane is the last component of aerobic respiration; at this level, more than 90% of the available O<sub>2</sub> is reduced directly to water by cytochrome oxidase through a series of redox reactions where electrons are passed rapidly from one component to the next, while less than 10% is reduced incompletely, leading to the formation reactive oxygen species (ROS) in aerobic conditions.

Superoxide anion ( $\bullet\text{O}_2^-$ ) results from O<sub>2</sub> reduction with one electron, and it is the most common oxidative free radical in human biology (Torres-Cuevas et al. 2017). O<sub>2</sub> reduction with two or three electrons forms hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxyl radical ( $\bullet\text{OH}$ ), respectively. Although H<sub>2</sub>O<sub>2</sub> is not structurally considered a free radical, it is much more reactive than molecular oxygen and is therefore included in the ROS group. The production of hydroxyl and hydroxide radicals is also triggered by the Fenton reaction, which involves ferrous ion and H<sub>2</sub>O<sub>2</sub>. By combining with nitric oxide (NO), ROS can generate peroxynitrite and other reactive nitrogen species (RNS), which further enhance free radical production due to their highly unstable chemical behavior.

While, at low concentrations, ROS and RNS can exert beneficial effects on physiological functions, such as immune regulation, smooth muscle relaxation, modulation of gene expression, and programmed cell death, at higher concentrations they can react harmfully with nearby proteins, membrane lipids, nucleic acids, or

other cell components, leading to structural modifications that can alter their function (Valko et al. 2007). In order to protect biological structures from the ensuing oxidative damage by maintaining a redox homeostasis, the levels of ROS and RNS in biological tissues are regulated by specific antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GP). Reduced glutathione further contributes to reduce both ROS concentration, either via direct interaction or serving as a cofactor for ROS-detoxifying enzymes (Lushchak 2012).

Oxidative stress ensues from an imbalance between free radical production on one side and their physiological clearance by antioxidant enzymes on the other and leads to acute and chronic detrimental effects on vital organs and tissues.

## Neonatal Hypoxic-Ischemic Encephalopathy: A Clinical Overview

Neonatal hypoxic-ischemic encephalopathy (HIE) is defined as a clinical syndrome of disturbed neurological function in the earliest days of life in the term infant, manifested by difficulty with initiating and maintaining respiration, depression of tone and reflexes, subnormal level of consciousness, and seizures resulting from an acute or subacute disruption of cerebral oxygen delivery (Nelson and Leviton 1991). Despite the advancements in perinatal care occurred over the past decades and the introduction of therapeutic hypothermia (TH) as a standard of care for neonatal HIE, this condition still represents a leading cause of neonatal mortality in low-income settings and of permanent neurologic disability in term neonates worldwide, with an estimated incidence that ranges from 1 to 8 per 1000 live births in high-income countries to as high as 26 per 1000 live births in low-income countries (Nelson and Leviton 1991; Lehtonen et al. 2017).

Antepartum risk factors for neonatal HIE include persistent occipital-posterior position, prolonged rupture of membranes, and maternal pyrexia. A sudden intrapartum event responsible for the acute decrease of fetal perfusion, such as placental abruption, uterine rupture, prolapse of the umbilical cord, or shoulder dystocia, is also often identified (Nelson et al. 2012; Douglas-Escobar and Weiss 2015). Repeated phases of fetal hypoxia due to reduced uterine perfusion during active labor contractions can further contribute to perinatal asphyxia. An additional although rarer condition associated with neonatal HIE is fetomaternal or fetofetal hemorrhage, which determines a chronic hypoxic state due to the reduced levels of fetal hemoglobin.

A pH <6.8 or a base excess <−20 mEq/l on a blood sample obtained from the umbilical artery, an Apgar score ≤ 3 at 10 min, the loss of the physiological fetal heart rate variability during labor, seizures occurring within the first 24 h from the insult, and evidence of multi-organ injury (i.e., increased transaminase, creatinine, creatinine kinase MB, and troponin T levels on blood tests) are strong predictors of the development of brain damage after perinatal asphyxia (Tonni et al. 2014). The individual characteristics of the neonate (e.g., gestational age, individual sensitivity to oxidative stress, metabolic, and cardiovascular status) together with the features of

the hypoxic-ischemic insult (i.e., chronic vs. acute, intermittent vs. persistent) further contribute to determine the severity of HIE. In term neonates, chronic or mild to moderate hypoxia-ischemia is typically associated with evidence of brain injury in the parasagittal watershed zones between anterior/middle and middle/posterior cerebral arteries, while a global and acute reduction of cerebral hypoperfusion results in injury to metabolically active tissues such as the basal ganglia, hippocampus, sensorimotor cortex, corticospinal tracts, and also the brainstem (Bano et al. 2017).

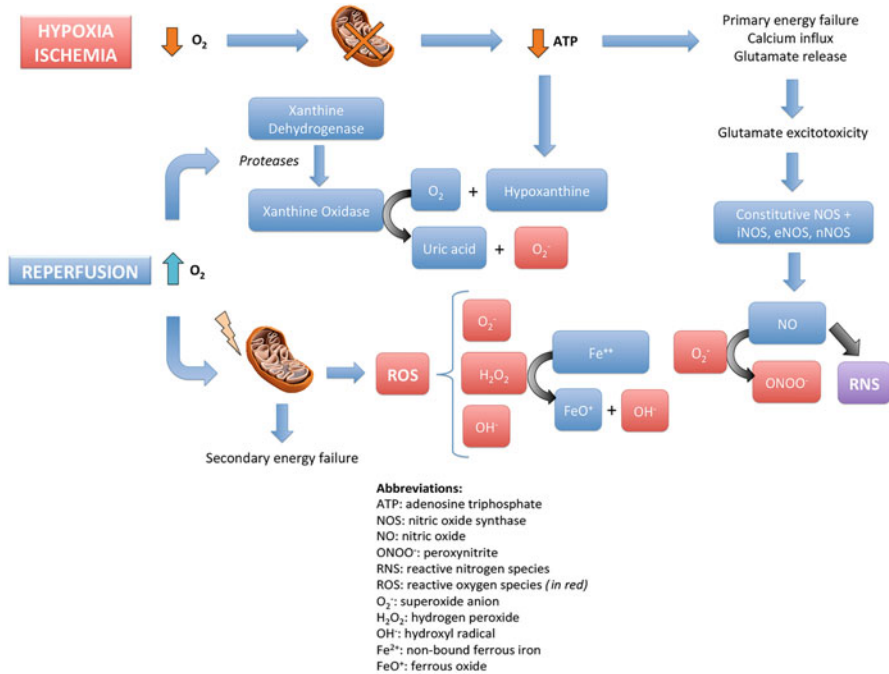
Following a hypoxic-ischemic insult, it is fundamental to monitor electrical brain activity with electroencephalography (EEG) or with amplitude-integrated EEG (aEEG) in order to detect abnormal electrical patterns or to identify seizures. Based on the combination of EEG/aEEG abnormalities and of specific clinical signs such as abnormal level of consciousness, reduced reflexes, and hypotonia, the severity of HIE can be assessed using the Sarnat grading scale (Sarnat and Sarnat 1976), which classifies the affected neonates into three stages of increasing severity (i.e., stage I, mild; stage II, moderate; stage III, severe). This staging system also entails a prognostic value: the higher the stage, the more severe the HIE, the higher the probability of major neurological sequelae.

Brain magnetic resonance imaging (MRI), especially if associated with spectroscopy or diffusion studies, provides important information to assess the extent of HIE-related brain damage and to predict long-term neurodevelopmental outcomes. A lower diffusion coefficient in the deep grey matter within the first 7 days following the hypoxic-ischemic insult is a predictor for poor neurodevelopment. Evidence of an abnormal signal intensity in the posterior limb of the internal capsule and/or in the basal ganglia is associated with an increased risk of mortality and of cerebral palsy (Ferriero 2004).

## The Role of Oxidative Stress in Neonatal HIE

The high oxygen requirements and the ample redox-active iron availability, together with the relatively low levels of antioxidants and the abundant contents of polyunsaturated fatty acids (PUFAs), make the infant brain particularly vulnerable to oxidative damage after a hypoxic-ischemic insult (Ferriero 2004). Cellular mechanisms underlying HIE are complex and involve a sequence of interconnected molecular events, such as oxygen deprivation, energy depletion, release of excitatory amino acids, and reperfusion that, as illustrated in Fig. 1, variously contribute to oxidative stress and to the ensuing cellular dysfunction and death.

In the first instance, acute cerebral hypoperfusion interrupts oxygen delivery to the brain, inhibiting oxidative phosphorylation in the mitochondria and shifting cellular metabolism from aerobic to anaerobic (Douglas-Escobar and Weiss 2015). Glucose utilization for anaerobic glycolysis is highly inefficient, and this, together with limited brain storages of glycogen, contributes to a rapid depletion of cerebral glucose, which represents the primary energy source for neural cells (Brekke et al. 2017). As a result of the decreased levels of adenosine triphosphate (ATP), ATP-dependent ion pumps are progressively inactivated, subsequently leading to



**Fig. 1** Molecular mechanisms leading to oxidative stress generation following hypoxia-ischemia and reperfusion. From Martini et al. (2020) with permission

intracellular accumulation of sodium and water, cell swelling, and necrotic cell death. The sequence of neural hypoxia, ischemia, and energy depletion culminates into cell membrane depolarization and release of glutamate, an excitatory amino acid. This process, defined as glutamate excitotoxicity, triggers an intracellular calcium influx that contributes to cell damage not only by exerting necrotic effects but also activates apoptotic cascades via N-methyl-D-aspartate (NMDA) and glutamate receptors. In order to induce a compensatory increase in cerebral blood flow via NO-mediated vasodilation (Iadecola 1997), glutamate excitotoxicity upregulates constitutive and inducible nitric oxide synthase (NOS). The resulting increase in NO production, however, further contributes to brain injury by boosting RNS and ROS production (Ferriero 2004; Hsu et al. 2014). The role of oxidative and nitrosative stress is particularly important in the development of deep grey nuclei injury, which is the brain lesion most frequently observed in term neonates following an acute ischemic insult (Ferriero 2004). The reason underlying the enhanced susceptibility of this area includes the presence of NOS-expressing (NOS+) striatal neurons that, in the developing brain, are relatively resistant to the noxious effects of hypoxia-ischemia and glutamate excitotoxicity (Ferriero et al. 1990). Following calcium influx and the activation of NMDA receptors, NOS+ neurons produce excessive amounts of NO which, in turn, is converted to peroxynitrite, a potent mediator of free radical damage (Barkhuizen et al. 2017). Due to their proximity to

such NOS<sup>+</sup> neurons, neural and glial cells located in the nearby striatal projections are exposed to a harmful bystander effect (McQuillen and Ferriero 2004). Evidence of reduced basal ganglia damage in association with selective NOS inhibition (Peeters-Scholte et al. 2002), after selective ablation of NOS<sup>+</sup> neurons (Ferriero et al. 1995) or following targeted disruption of the NOS gene (Ferriero et al. 1996) in animal models of hypoxia-ischemia, further supports this mechanism of injury.

Depending upon the timing and the efficacy of cerebral blood flow restoration, an energetic recovery progressively occurs after the hypoxic-ischemic insult, as proved by proton magnetic resonance spectroscopy showing near normal levels of ATP (Hope et al. 1987). Although reperfusion and reoxygenation are key for survival, they also pave the way to the so-called reperfusion injury in the perfusion-deprived and oxygen-depleted tissues, first described four decades ago (Cerra et al. 1975). This process is characterized by a paradoxical overproduction of ROS by complex I and III secondary to the resumption of oxidative phosphorylation in disrupted mitochondrial ETC (McCord 1985) and ultimately leads to secondary ATP depletion and subsequent apoptotic brain damage (Wyatt et al. 1989). One of the most important mechanisms of ROS production following reperfusion is related to the proteolytic conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO), which is boosted by the intracellular influx of calcium occurring in energy-depleted cells (Amaya et al. 1990). While XD utilizes hypoxanthine or xanthine as a substrate and NAD as a cofactor to produce NADH, XO is a superoxide-producing enzyme and, using O<sub>2</sub> as a cofactor, produces •O<sub>2</sub><sup>-</sup> and uric acid (UA) from the same substrates. This, together with the accumulation of hypoxanthine ensuing from ATP depletion during the hypoxic phase, remarkably enhances intracellular ROS production (Chung et al. 1997). Moreover, via the Fenton reaction, nonprotein bound iron (NPBI) and other transition metals available in the brain tissue further contribute to increase ROS levels exponentially (Valiko et al. 2005).

The excessive ROS production that follows the hypoxia-ischemia-reperfusion cycle further contributes to worsen the mitochondrial damage begun by primary ATP depletion, calcium influx, and glutamate excitotoxicity. This latent disruption of oxidative phosphorylation starts with reperfusion and usually lasts up to 6 h, finally leading to secondary energy failure (Douglas-Escobar and Weiss 2015). For this reason, the first 6 h following the hypoxic-ischemic event represent a useful, though narrow, therapeutic window to start TH, which decreases cerebral metabolism by reducing the whole body temperature to 33.5 °C, and for other neuroprotective strategies that are currently being tested in clinical trials, such as inhaled xenon, melatonin, erythropoietin, and allopurinol (Martini et al. 2020).

The phase of secondary energy failure is characterized by a delayed and progressive failure of oxidative metabolism, despite normal oxygenation levels. This phase usually starts from the first 6–8 h and lasts up to 72 h after the insult, and its extent depends upon the severity of the hypoxic-ischemic insult. ROS and RNS overproduction plays a central role in determining mitochondrial collapse, secondary cytotoxic edema, and neuroinflammation and in triggering multiple apoptotic pathways. Even after the secondary phase has resolved, however, ongoing effects on the brain can persist for several weeks to years; these effects include persistent

inflammation, impaired oligodendrocyte maturation and myelination, altered synaptogenesis, and epigenetic alterations, with relevant implications on long-term neurodevelopment (Davidson et al. 2021).

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## **Biomarkers of Oxidative Stress in Neonatal HIE**

The main clinical challenge after perinatal asphyxia is to identify infants at higher risk of developing moderate to severe HIE, who would therefore benefit from prompt neuroprotective treatments. As previously mentioned, the assessment of asphyxiated infants mainly relies on clinical, neurophysiological, and neuroimaging abnormalities. Nevertheless, the neurological status and the aEEG/EEG patterns of cerebral electrical activity can be significantly altered by sedatives, by anticonvulsants, and by TH itself (Thoresen et al. 2010), thus hindering to evaluate not only HIE severity, but also the response to ongoing treatments. Characteristic changes on brain MRI, particularly with conventional imaging assessments, may take several days to become apparent; moreover, unstable neonates may not tolerate either transport to the MRI scanner or the scan duration itself (Douglas-Escobar and Weiss 2015). Hence, the validation of blood, urine, and cerebrospinal fluid (CSF) biomarkers could contribute to support the sensitivity and specificity of clinical, aEEG/EEG, and neuroimaging findings for HIE severity and outcome prediction.

Among the biochemical parameters that have been currently proposed for the assessment of perinatal hypoxic-ischemic brain damage in clinical and research settings, it is possible to distinguish between markers of tissue injury, resulting from neuronal necrosis (e.g., neuron-specific enolase, matrix metalloproteinase, S100B protein, ubiquitin carboxyl-terminal esterase L1, etc.), gliosis (e.g., glial fibrillary acidic protein) or inflammation (e.g., interleukins), and markers of free radical production and related oxidative activity; this chapter will selectively focus on the latter group.

The gold-standard methods to assess the redox status in biological fluids are gas chromatography and liquid chromatography coupled to tandem mass spectrometry (GC-MS/MS, LC-MS/MS) (Torres-Cuevas et al. 2017). Together with thiobarbituric acid assays, these techniques can be used to measure urinary products of lipid peroxidation (Kuligowski et al. 2014). Moreover, the development of high- or ultra-performance LC-MS/MS has recently allowed to determine the concentration of ROS, RNS, and their metabolites in very small amounts of biological fluids (Cháfer-Pericás et al. 2015), and this is particularly important to translate the assessment of oxidative biomarkers to the neonatal population.

An important limitation hindering the routine clinical use of GC-MS/MS or LC-MS/MS resides in their high cost, complexity, and the need for trained specialists; for these reasons, the availability of these techniques is mainly limited to academic settings with research facilities (Torres-Cuevas et al. 2017).

The following paragraph provides a detailed overview on the available preclinical and clinical evidence on the main oxidative stress biomarkers in the context of neonatal HIE. A summary of the available biomarkers is provided in Table 1.



**Table 1** Summary of the main characteristics of the oxidative biomarkers investigated in the context of neonatal hypoxic-ischemic encephalopathy

Biomarker	Role in oxidative stress	Specimen				Proposed reference or cut-off values	Data during TH	Predictivity (HIE severity, outcomes)
		Cord blood, plasma/serum	Urine	CSF				
<b>MDA</b>	Lipid peroxidation marker	↑	↑	↑	Not available	Available	+	
<b>4-HNE</b>	Lipid peroxidation marker	↑	Not available	Not available	Not available	Not available	/	
<b>Isoprostanes (IP) Neuroprostanes (NP)</b>	Lipid peroxidation marker	↑8-IP	↑ NP	Not available	Normal cord IP values: <124.47 pg/mL	Not available	+(8-IP)	
<b>PC</b>	Protein oxidation markers	↑	Not available	Not available	Not available	Not available	+/-	
<b>AOPP</b>	Protein oxidation markers	↑	Not available	Not available	Normal cord values: <80.39 μmol/dL	Available	+(early assessments)	
<b>8-OHdG</b>	DNA peroxidation marker	↑	↑	↑	Not available	Available (plasma)	/	
<b>NPBI</b>	Substrate for Fenton reaction	↑	Not available	↑	Normal cord values: <6.91 μmol/L	Available (plasma)	+	
<b>SOD GP CAT</b>	Antioxidant enzymes	↑SOD ↑GP ↑CAT	Not available	↑SOD ↑GP ↑CAT	Not available	Available (plasma)	+(SOD and CAT)	
<b>Uric acid</b>	Catabolite of xanthine oxidase activity	Not available	↑	Not available	UA/creat ratio ≥ 2.3; Severe HIE and mortality	Not available	+	
<b>Nitric oxide</b>	Substrate for nitrosative species production	↑	Not available	↑	Not available	Not available	+	
<b>Total bilirubin</b>	ROS scavenger	↓	Not available	Not available	Not available	Available	+/-	

## Lipid Peroxidation Markers

Free radical species can insert oxygen molecules to lipids containing carbon-carbon double bonds, such as PUFAs or membrane phospholipids, to generate lipid peroxy radicals and hydroperoxides (Ayala et al. 2014). Following hypoxia-ischemia, free radical overproduction can overcome the adaptive upregulation of antioxidants enzymes, resulting in noxious peroxidative modifications of neuronal membranes. Once this oxidative process is initiated, a propagation of chain reactions generates a wide variety of lipid peroxidation products that can serve not only as biomarkers of global lipid peroxidation but, given the rich lipid composition of the developing brain, may also provide useful information to estimate the oxidative brain damage in neonatal HIE.

### Malondialdehyde

Malondialdehyde (MDA) is encountered among the most mutagenic products of the peroxidation of omega-3 and omega-6 fatty acids (Esterbauer et al. 1990) and has long been studied as a biomarker for this oxidative process (Ayala et al. 2014). Increased MDA levels in the cord blood of severely asphyxiated infants (Mondal et al. 2010; Mahmoud El Bana et al. 2016) and in the serum of newborns with HIE within the first 72 h of life (Singh et al. 1999; Thorat et al. 2004; Shouman et al. 2008; Kumar et al. 2008a; Mondal et al. 2010; Mutlu et al. 2015) have been consistently reported by currently available literature. Serum MDA concentration in the acute phase has also been shown to correlate with HIE severity (Thorat et al. 2004; Mutlu et al. 2015; Mahmoud El Bana et al. 2016); HIE infants who died or developed such complications as seizures, persistent neurological impairment, or evidence of brain lesions at neuroimaging showed the highest values of serum MDA (Yu et al. 2003; Shouman et al. 2008; Mondal et al. 2010).

MDA is water-soluble and, as such, is excreted in urines; hence, its excretion rate has also been investigated to evaluate lipid peroxidation extent in HIE. The ratio between urinary MDA and creatinine (uMDA/uCr) increases significantly over the first 24–48 h of life in asphyxiated neonates (Siciarz et al. 2001; Banupriya et al. 2008; Mahmoud El Bana et al. 2016), showing a positive correlation with Sarnat stage and a negative correlation with Apgar score (Banupriya et al. 2008; Mahmoud El Bana et al. 2016). Furthermore, a higher uMDA/uCr ratio was seen in HIE neonates who died compared to survivors; in this regard, a cut-off level of 3.495 µg/mg has been proposed to predict death following perinatal asphyxia with a sensitivity and specificity of 87.5% and 91.7%, respectively (Banupriya et al. 2008).

In the context of HIE, however, serum and urinary MDA are not specific for the lipid peroxidation processes within the brain. MDA concentration in CSF has the advantage of a higher specificity for the cerebral tissue compared to serum or urine and could therefore provide a better estimate of oxidative brain injury in neonatal HIE. Significantly higher MDA levels in CSF have been reported between 24 and 48 h of life in asphyxiated term infants who developed HIE compared to controls (Kumar et al. 2008b) and in HIE infants who expired or developed neurological

deficits compared to those with normal neurological status at hospital discharge (Shouman et al. 2008). Within each category, however, MDA levels in CSF were much lower than in plasma and, for increasing HIE stages, showed smaller surges, resulting in a paradoxically diminished ratio of CSF/plasma MDA in infants progressing to Sarnat stage III compared to controls and in fatal cases compared to survivors (Kumar et al. 2008b). Larger data are further required to validate CSF MDA as a possible marker for oxidative brain damage in neonatal HIE.

#### **4-Hydroxynonenal (HNE)**

4-hydroxynonenal (4-HNE) derives from peroxidation of omega-6 fatty acids and can either act as a signalling molecule modulating cell apoptosis or exert cytotoxic effects with long-lasting biological consequences (Esterbauer et al. 1990). Evidence on its role as a lipid peroxidation marker after perinatal asphyxia is limited to one study by Schmidt et al. (Schmidt et al. 1996), who analyzed 4-HNE levels in cord blood samples from preterm and term neonates. 4-HNE increased with increasing gestational age and rose significantly after the hypoxic insult, reflecting sensitively the extent of lipid peroxidation. However, while evidence on other lipid peroxidation products has progressively grown over the last two decades, no further data are so far available to add knowledge on 4-HNE as a biomarker of oxidative stress in HIE. This may be due to the unstable nature of 4-HNE that contributes to the technical complexity of its determination.

#### **Prostaglandin-like Peroxidation Products (PPPs)**

Prostaglandin-like compounds derived from free radical-catalyzed peroxidation of arachidonic and docosahexaenoic acid include isoprostanes, isofurans, neuroprostanes, and neurofurans. High brain levels of these compounds have been documented in animal models of global perinatal asphyxia (Calamandrei et al. 2004; Solberg et al. 2017) and in term, asphyxiated infants at the autptic evaluation (Back et al. 2005).

Among PPPs, F2-isoprostanes have proved to reliably reflect the extent of oxidative processes after hypoxia-ischemia-reperfusion (Sakamoto et al. 2002), and the development of an ultra-performance LC-MS/MS, sensitive to very small amounts of serum, has allowed to investigate their role as lipid peroxidation biomarkers in the context of perinatal asphyxia (Cháfer-Pericás et al. 2015). Higher levels of 8-isoprostane and of total isoprostanes were detected in cord blood samples from acidotic and depressed infants compared to healthy neonates (Cháfer-Pericás et al. 2016); in particular, 8-isoprostane positively correlated with the severity of perinatal asphyxia, defined according to cord gas pH, Apgar score, and neurological status at birth. However, the evaluation of serum F2-isoprostanes levels over the first 5 days in HIE infants treated with TH failed to demonstrate different concentrations in relation to the severity of HIE or of brain damage extent at neuroimaging (Negro et al. 2018).

Recently, a urinary panel including multiple PPPs has been tested to estimate oxidative processes during the first 5 days of life in term HIE infants undergoing TH (Cascant-Vilaplana et al. 2021). No difference in the urinary concentration of isoprostanes, isofurans, neurofurans, and neuroprostanes, except of 14(RS)-14-F<sub>4t</sub>-neuroprostane, was detected over the study period, while significantly different levels of 14(RS)-14-F<sub>4t</sub>-neuroprostane, total isoprostanes, 15(RS)-15-F<sub>2t</sub>-isoprostane, and total dihomio-isoprostanes were observed in relation to specific MRI patterns of brain damage.

Given the heterogenic findings of currently available literature, further data are necessary to establish the feasibility of PPPs and, in particular, of F<sub>2</sub>-isoprostanes, as oxidative biomarkers in neonatal HIE.

## Protein Oxidation Markers

The free radical overload generated by hypoxia-ischemia-reperfusion can lead to carbonylation, fragmentation, nitration, cross-linking, and loss of thiol groups in cell proteins. Among the ensuing oxidation products, protein carbonyls (PC) and advanced oxidation protein products (AOPP) have been proposed as possible biomarkers of protein oxidation processes in neonatal diseases, including HIE (Mondal et al. 2010; Perrone et al. 2012).

A rise in PC concentration on cord blood and in serum samples after 48 h from the hypoxic-ischemic insult has been reported in asphyxiated term neonates (Mondal et al. 2010). Although PC levels did not differ significantly in relation to Sarnat staging or developmental outcome at 9 months, HIE infants who developed seizures showed a higher concentration at 48 h compared to those who did not (Mondal et al. 2010).

As for AOPP, Buonocore et al. (2000) have analyzed the concentration of these markers in cord blood from normoxic and hypoxic preterm infants, observing increased levels in the latter group and a significant positive correlation with plasma hypoxanthine and total hydroperoxide levels. In a pre-TH study, increased AOPP levels were reported on cord blood and at 48 h of life in term HIE infants compared to controls. In term asphyxiated infants undergoing TH, however, AOPP levels were significantly higher only at 4–6 h of life in severe compared to mild-moderate HIE infants, whereas no difference between HIE stages was observed at later evaluations, ranging from 24 h to 5 days of life (Negro et al. 2018). Similarly, no significant difference in serum AOPP levels on day 1 (>6 h) and 5 was reported by Mutlu et al. (2015) between term HIE infants treated with TH and controls, although a trend toward increased levels in the HIE group was observed. Given the evidence of higher AOPP levels in untreated HIE infants compared to controls and, in studies performed in the TH era, prior (i.e., 4–6 h), but not during and after this treatment, a beneficial effect of TH in reducing protein oxidation processes may be hypothesized to explain these findings.

In their recent study, Cascant-Vilaplana et al. (2021) used a LC-MS/MS-based urinary panel of oxidative biomarkers, inclusive of several compounds derived from

protein oxidation, to evaluate the extent of oxidative processes in HIE infants. The urinary concentration of protein oxidation biomarkers increased significantly throughout the first 5 days of life and showed significantly different levels in relation to specific MRI patterns of brain injury. A significant, independent association between serum AOPP levels during the first 5 days of life and the extent of hypoxic-ischemic brain injury, assessed using a validated MRI score, was also reported by Negro et al. (2018); this association was stronger in males infants, thus suggesting a possible gender-related susceptibility to oxidative neurological damage.

## DNA Peroxidation Markers

The oxidized DNA nucleoside 8-hydroxydeoxyguanosine (8-OHdG) results from the harmful peroxidative changes to nucleic acids and, over the past decade, has been investigated as a potential biomarker for oxidative DNA damage in preterm and term neonates (Matsubasa et al. 2002; Fukuda et al. 2008; Gane et al. 2014; Bandyopadhyay et al. 2017; Cascant-Vilaplana et al. 2021). With regard to HIE, however, little data are available. In 2008, Fukuda et al. evaluated urine and CSF 8-OHdG concentration in a cohort of children with various types of brain damage, including a small subgroup of neonates with HIE, who showed significantly higher CSF and urinary 8-OHdG levels compared to control subjects (Fukuda et al. 2008). However, data on the timing of collection of CSF and urinary specimens in the HIE subgroup was not specified, and these, together with the small study sample, are potential limitations to these study results.

Of interest, Gane et al. (2014) examined the impact of TH on blood levels of 8-OHdG in treated vs. untreated HIE infants before this treatment became a standard of care worldwide. While pre-treatment levels were similar between the two study groups, untreated infants showed a significantly higher concentration of 8-OHdG compared to the treated group at 36 h of life and after the completion of the hypothermic treatment, thus supporting the effectiveness of TH in reducing the oxidative burden.

8-OHdG was also included among the urinary oxidative biomarkers recently evaluated in HIE neonates undergoing TH; according to this study results, the urine concentration of this biomarker increased significantly over the first 5 days of life despite the hypothermic treatment (Cascant-Vilaplana et al. 2021).

## Antioxidant Enzymes

As previously specified, the main antioxidant enzymes involved in the regulation of the redox homeostasis and in the defense from oxidative damage include SOD, CAT, and GP. After the occurrence of a hypoxic-ischemic insult, the activity of these enzymes is significantly enhanced to counteract the overproduction of free radicals and their harmful effects. Consistently, significantly increased levels of SOD, GP

and CAT have been reported in the cord blood of term asphyxiated neonates who developed HIE compared with healthy controls (Singh et al. 1999; Kumar et al. 2008b; Bharti et al. 2009). SOD and CAT concentration in cord blood samples also showed a significant association with Sarnat stages (Kumar et al. 2008b), suggesting that the early upregulation of antioxidant enzymes may effectively reflect the severity of HIE, with potential prognostic implications.

At 24 h, blood concentration of SOD, but not of GP, was found to be significantly increased in infants with mild and moderate HIE compared to controls (Thorat et al. 2004; Mutlu et al. 2015). Furthermore, consistently with the progressive consumption of antioxidant capacities after the acute oxidative burst, a trend toward a decrease in plasmatic SOD levels from day 1 to 5 after perinatal asphyxia has also been reported; on day 5, however, SOD concentration in blood samples from HIE infants was still significantly higher compared to the control group (Mutlu et al. 2015).

With regard to antioxidant enzymatic activities in other biological fluids such as CSF, which may increase the sensibility toward the oxidative processes ongoing in the brain, current data are limited to Gulcan et al. (2005), who evaluated SOD, GP, and CAT activity in the CSF of full-term asphyxiated neonates over the first 72 h of life. According to their findings, SOD activity was significantly higher in HIE infants versus controls, whereas a significant increase of GP and CAT activity was observed only in neonates with severe HIE compared to mild HIE and to the control group. However, this study was performed in the pre-TH era; therefore these results require further confirmation on treated HIE cohorts.

Recently, a possible association between HIE sequelae and specific functional polymorphisms of manganese SOD2, GP1, and CAT genes has been investigated. No difference in SOD2, GP1, and CAT genotype distribution between HIE infants developing epilepsy and controls was observed (Esih et al. 2017), whereas CAT rs1001179 polymorphisms resulted significantly associated with the development of cerebral palsy (Esih et al. 2016), thus hypothesizing a possible role for this polymorphism in identifying highly susceptible asphyxiated infants.

## Uric Acid

The restoration of adequate O<sub>2</sub> supplies following a hypoxic-ischemic insult enhances the conversion of XD to XO, which uses hypoxanthine or xanthine as substrates and O<sub>2</sub> as a cofactor to generate •O<sub>2</sub><sup>-</sup> and UA. Hence, by serving as a proxy for XO activity, the concentration of UA in biological fluids may reflect the extent of the ensuing ROS production and has therefore been proposed as an economical and easily accessible oxidative biomarker. Being water-soluble, UA is excreted by the kidney; hence, urinary levels of UA have been largely evaluated as noninvasive biomarkers for free radical production following perinatal asphyxia.

Current evidence is consistent in reporting an increased ratio between urinary UA and urinary creatinine (uUA/uCr) in both term and preterm asphyxiated

newborns compared to controls within the first 48–72 h of life (Chen et al. 2000; Banupriya et al. 2008; Basu et al. 2008; Bhongir et al. 2015; Mahmoud El Bana et al. 2016; Patel et al. 2017). This ratio has shown a significant association with both Apgar score (Banupriya et al. 2008; Basu et al. 2008; Bhongir et al. 2015) and Sarnat stage (Akisü and Kültürsay 1998; Banupriya et al. 2008): the lower the Apgar score, the more severe the HIE, the higher the urinary excretion of UA. Cut-off levels of  $\text{uUA/uCr} \geq 2.3$  have been proposed to be reliably diagnostic of HIE and to predict the related mortality with good sensitivity and specificity in term asphyxiated infants born in a low-resource setting (Banupriya et al. 2008; Patel et al. 2017). However, the cohorts on which this cutoff was determined were not treated with TH; therefore this data needs to be confirmed on cooled infants to ascertain possible effects of the hypothermic treatment on  $\text{uUA/uCr}$  ratio.

### Nonprotein-Bound Iron

The hypoxia-ischemia-reperfusion cycle and the ensuing inflammation triggers the release of NPBI from erythrocyte hemoglobin. In turn, through the Fenton reaction, NPBI interacts with  $\bullet\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  to form highly reactive  $\bullet\text{OH}$ , which enhances protein (Marzocchi et al. 2005) and lipid oxidation (Signorini et al. 2008). The first report on NPBI as an OS biomarker in perinatal asphyxia dates 20 years back, when Dorrepaal et al. (1996) observed an increased prevalence of detectable plasma NPBI with increasing HIE severity in asphyxiated infants >34 weeks' gestation. In a recent study on HIE infants undergoing TH, increased levels of plasma NPBI were observed at 4–6 h, but not later, in neonates with severe compared to mild-moderate HIE (Negro et al. 2018), suggesting a potential role of TH in dampening down the related oxidative stress.

A potential predictive role for NPBI levels on HIE neurodevelopmental outcomes has also been proposed. Dorrepaal et al. noticed that three out of four severely asphyxiated neonates with a normal neurological outcome at 1 year of age had no detectable NPBI plasma levels during the first 8 h after birth (Dorrepaal et al. 1996). In a similar fashion, higher levels of plasma (Yu et al. 2003; Shouman et al. 2008) and CSF NPBI (Shouman et al. 2008) within the first 72 h of life were observed in neonates with moderate or severe HIE who died or developed an abnormal neurological status compared to those who were neurologically normal at discharge. However, these data were collected before TH introduction and thus require further validation in treated HIE cohorts.

Of note, while a cut-off value of NPBI to identify HIE infants has not been defined yet, reference intervals for cord blood NPBI have been recently investigated, and a cut-off value of  $6.91 \mu\text{mol/L}$  has been proposed as the upper normal threshold in healthy, non-asphyxiated term infants (Longini et al. 2017).

## Nitric Oxide

The upregulation of NOS expression triggered by hypoxia-ischemia enhances the production of NO, which acts as a free radical and reacts with other substrates to form peroxynitrite and other RNS. As such, NO concentration and the nitrates/nitrites ratio, which serves as a proxy for NO levels, have been investigated in the context of neonatal HIE. In several studies performed in pre-hypothermia years, increased plasma levels of NO in plasma (Shi et al. 2000; Thorat et al. 2004) and CSF samples (Gunes et al. 2007), as well as higher plasma nitrates/nitrites ratio (Kumar et al. 2008a), have been detected within the first 24 h of life in neonates with HIE compared to controls. A positive correlation between the plasmatic and CSF concentration of NO and HIE severity, expressed by increasing Sarnat staging, was observed (Shi et al. 2000; Thorat et al. 2004; Gunes et al. 2007). Moreover, plasma NO was much higher in HIE infants with neuroradiological evidence of brain damage compared to those with no abnormalities at brain MRI (Shi et al. 2000). Although these data are consistently supportive of the role of NO as a biomarker for OS and clinical severity in HIE, the possible effect of TH still needs to be evaluated.

## Bilirubin

Despite its long-established toxicity on the cerebral tissue at high concentrations, unconjugated bilirubin is also endowed with antioxidant properties (Stocker et al. 1987). In particular, it can act as a highly efficient ROS scavenger in conditions of oxidative stress and may serve as a reducing substrate for peroxidases in the presence of H<sub>2</sub>O<sub>2</sub> or other organic hydroperoxides (Vitek and Ostrow 2009). Hence, also considering the low costs and wide availability of its assessment, serum total bilirubin (sTB) levels in neonatal HIE have been investigated. Preliminary data from a retrospective study on HIE cohorts have shown an inverse correlation between lactate concentration, which reflect the extent of perinatal hypoxia-ischemia (Shah et al. 2004), and sTB (Haga et al. 2020). Consistently, lower peak and mean sTB levels were observed in HIE infants with moderate to severe HIE compared to controls, independently of ongoing TH, throughout the first 5 days after birth (Bin-Nun et al. 2018; Dani et al. 2018). These results are consistent with the scavenging role of bilirubin, which can be therefore consumed following perinatal asphyxia, and the degree of sTB consumption is likely associated with the severity of HIE.

## Galectin-3 and Quinolinic Acid

Galectin-3 (Gal-3) is a  $\beta$ -galactoside-binding lectin produced by activated tissue macrophages and microglial cells (Liu et al. 1995) that, among its functions, enhances ROS production by activating NADPH oxidase in primed inflammatory cells (Karlsson et al. 1998). Microglia and macrophages also produce quinolinic acid



(Quin), a neurotoxic metabolite of L-tryptophan that, acting as a NMDA receptor agonist, triggers not only excitotoxic brain damage (Schwarcz et al. 1983) but also the oxidative burst (Santamaría et al. 2001).

In 2013, Savman et al. investigated CSF levels of Gal-3 and Quin in relation to perinatal asphyxia, observing increased levels of both biomarkers in asphyxiated infants compared to controls (Sävman et al. 2013). In addition, Gal-3 was significantly higher in HIE infants who died or developed neurological sequelae compared to those with normal outcome. In order to evaluate their specificity, the authors measured Quin and Gal-3 levels also in a cohort of non-asphyxiated septic infants, showing no difference with healthy controls. This encouraging preliminary evidence paves the way to a wider evaluation of Gal-3 and Quin validity as potential OS biomarkers in neonatal HIE.

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## **Diagnostic and Prognostic Applications of Oxidative Biomarkers in Neonatal HIE**

This chapter has reviewed the available evidence on the oxidative and nitrosative biomarkers that have been investigated to assess the extent of brain injury following a hypoxic-ischemic insult in the neonatal population.

While data on oxidative biomarkers that have more recently come into the spotlight (e.g., PPPs, gal-3, Quin) are very preliminary, other biomarkers, such as MDA, NPBI, UA, and NO, have been long investigated in the context of perinatal asphyxia, providing encouraging evidence for the identification of asphyxiated neonates at higher risk for brain injury, especially if evaluated in early phases (e.g., cord blood or 4–6 h of life). Since TH needs to be undertaken within the first 6 h after the hypoxic-ischemic insult to guarantee its therapeutic potential, the development of oxidative biomarker panels on easily available samples (i.e., urine or blood) may aid to identify infants at higher risk of brain injury or of HIE progression that would benefit from treatment. Although CSF biomarkers may better reflect the extent of oxidative brain injury compared to other biological fluids, the need to perform an invasive maneuver such as a lumbar puncture reduces their clinical potential in asphyxiated neonates.

The translation of oxidative biomarkers to routine neonatal practice, however, has not been achieved yet, due to the following important limitations. First, despite the development of ultra GC-MS/MS or LC-MS/MS has allowed to determine several oxidative biomarkers on very small biological samples, the high costs and the complexity of these techniques limit their availability and have likely contributed to the small study samples on which these biomarkers have been tested. Hence, further data on larger cohorts are required to validate the biomarkers reviewed in this chapter and to try to establish possible reference intervals. Moreover, the introduction of TH as a standard of care has split the available literature into pre- and post-TH epochs. By decreasing cerebral metabolism, TH contributes to reduce the oxidative burst that follows a hypoxic-ischemic hit and, as such, may influence the levels of oxidative biomarkers. Therefore, data obtained from untreated infants after the first

6 h need to be confirmed during TH, to rule out a reduction of the diagnostic and prognostic biomarker potentials while this treatment is ongoing. Finally, it has been shown that several conditions, either antenatal (sepsis, maternal preeclampsia, maternal tobacco, etc.) or postnatal (sepsis, meconium aspiration, etc.), can alter the neonatal redox homeostasis and should be thus taken into account when the oxidative status of asphyxiated neonates is investigated.

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## Mini-Dictionary of Terms

- **Hypoxic-ischemic encephalopathy (HIE):** clinical syndrome of disturbed neurological function resulting from an acute or subacute disruption of cerebral oxygen delivery.
- **Reactive oxygen species (ROS):** highly reactive chemical compounds derived by incomplete O<sub>2</sub> reduction or by redox reactions.
- **Oxidative stress:** condition characterized by an enhanced ROS production that overcomes the antioxidant capacities of the organism and causes detrimental effects on biological structures.
- **Sarnat stage:** staging system for HIE severity classification (stage I, mild; stage II, moderate; stage III, severe) based upon specific clinical and electroencephalographic features.
- **Therapeutic hypothermia (TH):** standard treatment for neonatal HIE, characterized by whole body cooling to 33.5 °C starting within the first 6 h following the hypoxic-ischemic insult and continuing for 72 h.

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## Key Facts of Oxidative Biomarkers in Neonatal HIE

- **Lipid peroxidation biomarkers:** this group of biomarkers has been most extensively investigated in neonatal HIE, providing consistent evidence on their diagnostic and prognostic value; data on CSF samples are also available.
- **Protein oxidation biomarkers:** limited, although promising evidence for early assessments in neonatal HIE; possibly influenced by TH. Normal cord blood ranges available.
- **Nonprotein-bound iron:** increased in HIE infants; the predictive value on HIE severity needs to be validated during TH. Normal cord blood ranges available.
- **Uric acid (UA):** urine UA concentration is easily and noninvasively available and may represent a promising oxidative biomarker in neonatal HIE. A urinary UA/creatinine ratio  $\geq 2.3$  may predict severe HIE and the related mortality.
- **Antioxidant enzymes:** correlation between cord blood and HIE severity; more heterogeneous evidence on blood levels over the first 5 days of life. Limited data on CSF specimens.

## Summary Points

- Given the key role of oxidative stress in the development of brain injury following a hypoxic-ischemic insult, multiple oxidative biomarkers have been explored in the context of neonatal HIE, with the aim to implement available diagnostic and prognostic tools.
- Serum biomarkers may have a low specificity to effectively estimate the extent of oxidative processes underlying brain injury, as they may rather reflect systemic oxidative stress.
- CSF specimens may increase the diagnostic value of oxidative biomarkers toward the development of neonatal HIE; however, their collection require invasive maneuvers, and the available evidence on CSF biomarkers is still limited.
- Data on oxidative biomarkers obtained before the introduction of TH as a standard treatment for neonatal HIE may not be applicable to cooled infants, since this treatment contributes to reduce the oxidative burden during the secondary energy depletion.
- Validating these biomarkers in relation to the available neuroprotective treatments and, in particular to TH, may add useful information also to monitor the efficacy of these treatments in dampening down oxidative stress.
- The high costs, the technical complexity, and the need for trained personnel associated with GC-MS/MS or LC-MS/MS have limited their availability; hence, despite encouraging evidence, oxidative biomarkers are not routinely used in neonatal clinical settings yet.

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