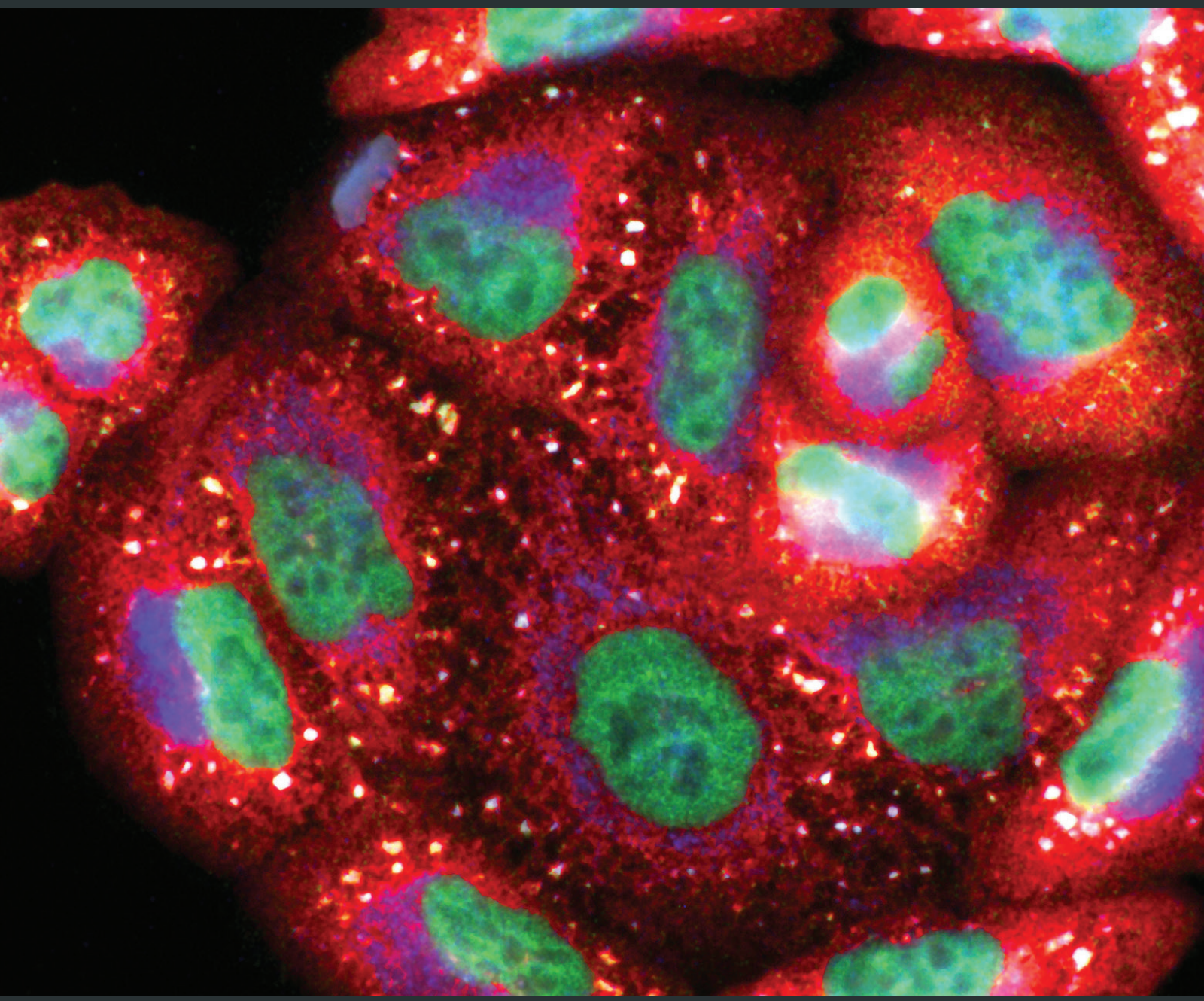
Oxidative Medicine and Cellular Longevity

Role of Oxidative Stress in Maternal and Neonatal Diseases

Lead Guest Editor: Carlo Dani
Guest Editors: Fabio Mosca, Diego Gazzolo, Federico Mecacci, Irene Cetin, and Giuseppe Buonocore



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Editorial

Role of Oxidative Stress in Maternal and Neonatal Diseases

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The objective of this special issue is to address correlations between oxidative stress and neonatal diseases. Many papers were submitted, and after a thorough peer review process, eight papers were selected to be included in this special issue. These research works provide relevant insights toward better understanding of pathophysiology of some preterm infants' complications and obesity in pregnancy. We believe that published papers via this special issue introduce readers to the latest advances in the field.

The paper by N. Laforgia et al. discusses the role of oxidative stress in the pathophysiology of congenital malformations. Oxidative stress might disrupt signalling pathways with a causative role in birth defects. New insights in the knowledge of these mechanisms of oxidative stress-related congenital malformations represent the basis of possible clinical applications in screening, prevention, and therapies.

The paper by C. Mandò et al. reports that lipotoxic placental environment changes the mitochondrial function, with excessive production of reactive oxygen species in maternal obesity, with increased inflammation and oxidative stress. In obese pregnancies, maternal glycemia or the maternal nutritional status and lifestyle might affect the pattern of mitochondrial dysfunction and possibly affect pregnancy outcomes.

The paper by C. Poggi and C. Dani presents data supporting the oxidative stress involvement in detrimental pathways activated during neonatal sepsis, eventually leading to organ dysfunction and death. Moreover, they discussed the possible role of antioxidant treatment during neonatal sepsis, including melatonin and pentoxifylline, or novel antioxidant molecules, such as edaravone and endothelin receptor antagonists, which are at present under investigation in animal models.

The paper by S. Perrone et al., details that newborns are particularly susceptible to OS and oxidative damage due to the increased generation of free radicals and the lack of adequate antioxidant protection.

They provide an update on the pathogenesis of the so-called "free radical-related diseases of prematurity," including retinopathy of prematurity, bronchopulmonary dysplasia, intraventricular hemorrhage, periventricular leukomalacia, and necrotizing enterocolitis.

The paper by A. Aceti et al. examines the role of oxidative stress (OS) in the pathogenesis of neonatal necrotizing enterocolitis and explores potential preventive and therapeutic antioxidant strategies. They discuss the protective effect of maternal milk exploring the so-called "milky way" for reducing oxidative stress by implementing human milk feeding and the risk of developing necrotizing enterocolitis. Other

possible prophylactic strategies, such as the use of melatonin and lactoferrin, are discussed.

The paper by Silberstein et al. considers that women with preeclampsia suffer from acute oxidative stress and high lipid oxidation in plasma. Therefore, the authors compared levels of polyphenols and lipid peroxidation in colostrum of nursing mothers with and without preeclampsia. They found that polyphenols were higher and lipid oxidation was lower in colostrum of women with preeclampsia, as if it exists a maternal compensation mechanism that protects the newborn from the stress processes the mother experiences. The paper by S. Negro et al. is aimed at evaluating the predictive role of a panel of oxidative stress biomarkers for the early identification of infants at high risk of HIE and their validation through the correlation with MRI findings. Advanced oxidation protein products (AOPP), nonprotein-bound iron (NPBI), and F2-isoprostanes (F2-IsoPs) were measured. Newborns with severe asphyxia showed higher oxidative stress than those with mild asphyxia at birth. AOPP are significantly associated with the severity of brain injury assessed by MRI, especially in males.

The paper by M. C. Pintus et al. evaluated the possible role of metabolomics in diagnosing bronchopulmonary dysplasia (BPD) in a cohort of preterm infants. They found that the discriminant urinary metabolites were alanine, betaine, trimethylamine-N-oxide, lactate, and glycine. They conclude that utilizing metabolomics, it is possible to detect in the first week of life infants who subsequently developed BPD.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this special issue.

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Research Article

Colostrum of Preeclamptic Women Has a High Level of Polyphenols and Better Resistance to Oxidative Stress in Comparison to That of Healthy Women

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Preeclampsia is a common pregnancy complication. Abnormal development of the placenta is the prevailing cause theory of this complication. Women with preeclampsia suffer from acute oxidative stress and high lipid oxidation in plasma. The aim of this study was to compare levels of polyphenols and lipid peroxidation in colostrum of nursing mothers with and without preeclampsia. The study was conducted at the Department of Obstetrics and Gynecology at Soroka University Medical Center. The study group consisting of 18 women, who were diagnosed with preeclampsia, was compared to the control group: 22 healthy women. The total phenolic content in the colostrum was determined by using the Folin–Ciocalteu method. Lipid peroxidation was determined by measuring MDA, using the TBARS assay. Polyphenol concentrations were significantly higher (about 33%) in the colostrum of the study group compared with the control group ($p = 0.00042$). Lipid peroxidation levels (MDA) were significantly lower (about 20%) in the colostrum of the study group compared with the control group ($p = 0.03$). Negative correlation was found between MDA concentration and the polyphenol level ($R = -0.41$, $p = 0.02$). In conclusion, we showed in this study a potential compensation mechanism that protects the newborn of a mother with preeclampsia from the stress process experienced by its mother.

1. Background

During pregnancy, there is an increase in the oxidative stress, a process created by a normal systemic inflammatory response; this results in high volumes of circulating reactive oxygen species (ROS). The placenta is the main source of ROS during pregnancy [1–3]. The oxidative stress formed during pregnancy is counteracted by the increased synthesis of antioxidants.

Preeclampsia is a common pregnancy complication that occurs in about 4% of pregnancies. There are two degrees of severity for the preeclampsia syndrome according to the symptoms, physical examination, and laboratory results: mild preeclampsia and severe preeclampsia. The signs and symptoms of preeclampsia are high blood pressure, high concentration of protein in the urine (kidney damage), severe headaches, visual impairment (blurring, temporary vision loss, or strong sensitivity to light), abdominal pain, an increase in the level of liver enzymes indicating liver damage, oliguria and anuria, shortness of breath, laboratory disturbances, preterm labor, and placental abruption. In most cases, some or all symptoms occur after the 20th week [4]. This complication is thought to be abnormal development of the placenta, mostly due to insufficient remodeling of the maternal vasculature perfusing the intervillous space. This may lead to a complex process of ischemia-reperfusion in the placenta with the release of cytotoxic factors into the maternal circulation. The uteroplacental hypoperfusion during preeclampsia increases oxidative stress in both the mother and the fetus [5–8].

The oxidative stress in placental cells is created by free radicals released from the inadequately perfused fetoplacental

unit. The plasma membranes of the circulating blood cells can be oxidized when passing through the ischemic placenta contributing to the propagation in this way of the oxidative stress to distal tissues. The antioxidant protection is reduced in preeclampsia, because of a decrease in free radical scavengers and the activity of the antioxidant enzymes [9].

In the biochemical aspect, previous studies have shown that women with preeclampsia suffer from oxidative stress and high lipid oxidation in plasma and lack some antioxidant groups [10].

Polyphenols represent a group of chemical substances that is common in plants, and it is structurally characterized by the presence of one or more phenol units. Polyphenols are the most abundant antioxidants in human diet. The largest and best studied class of polyphenols is flavonoids which include several thousand compounds [11]. The activity of polyphenols as antioxidants is varied: breaking chain reactions involving free radicals, suppressing the formation of free radicals in the cell, and chelating (binding to metallic ions) the free metal ions involved in the creation of radicals [12].

Very few studies have investigated the differences in the composition of colostrum of healthy women, compared with women who suffered from preeclampsia. One research showed that milk LCPUFAs and neurotrophins are altered in preeclampsia. They suggested that LCPUFA could plausibly influence the growth especially in children born to mothers with preeclampsia [13]. It was also shown that in preeclampsia, high cytokine levels in breast milk persist at least for 30 days. These results suggest that preeclampsia may affect milk cytokine balance and offer an immunological signal for the host defense in high-risk neonates [14]. Despite the great importance of polyphenols in the diet, there have been no studies that measured the content of polyphenols in colostrum. In addition, even though polyphenols have potential pathophysiological significance in preeclampsia, we could not find a study that measured their levels in colostrum of women who suffered from preeclampsia in comparison to healthy women colostrum.

The aim of this study was to compare levels of polyphenols and lipid peroxidation in colostrum of nursing mothers with and without preeclampsia.

2. Materials and Methods

2.1. Sample Collection for Research. Colostrum collection was performed at the Soroka University Hospital in the Department of Obstetrics. A total of 40 nursing women participated in the study and were divided into two groups: the control group consisted of healthy pregnant women ($n = 22$) and the study group consisted of pregnant women who were diagnosed with mild (4) or severe preeclampsia (14) (total $n = 18$). However, the mild preeclamptic women were almost severe, and we decided to analyze them with the severe group. Women with preeclampsia usually stay after delivery in the delivery room for close follow-up and treatment. Usually, their situation is not easy, and due to that and the fact that they want their milk for the baby, it is not easy to recruit them for the study. The sample sizes were decided to be around 20, a number that can allow us to do good statistical analysis and is achievable. Since in our hospital around 50% of the parturient are Bedouins and 50% are Jews, we are very familiar with the Arabic language and with this population. Recruitment of the women to the study was done before they gave birth. After having discussion with the women, the following procedures were performed. After washing hands and cleaning the nipple, the women pressed and squeezed the nipple for colostrum. This was done in the presence and guidance of the physician that recruited the woman. Primary milk, colostrum, was collected during the period of day one to day 7 after delivery. Milk samples were kept in the refrigerator at a temperature of −20°C until assays were performed. The collection of samples for research purposes was approved by the Ethics Committee of Soroka Hospital, in accordance with the Helsinki declaration. All the women recruited for the study signed on a document of informed consent. The women were asked to characterize their diet (vegetarian vs. regular) and whether they smoke cigarettes or consume alcohol.

2.2. Determination of Total Polyphenol Content in Colostrum. The total phenolic content in the colostrum was determined by using the Folin–Ciocalteu method [15]. This method is based on the redox reaction of the reagent forming a blue color pigment with typical absorbance at 760 nm. All UV-Vis measurements were performed using a Cary 100 Bio, UV-Visible spectrophotometer.

2.3. Extraction of Polyphenols from Colostrum. In order to extract the polyphenol from the colostrum, we developed optimum condition for extraction. The goal was to extract maximum polyphenols, while at the same time receiving a clear solution without proteins. 1.0 ml of colostrum was added to a 10 ml polypropylene centrifuge tub, followed by adding 1.0 ml of pure water, 2.0 ml of pure ethanol, 0.6 ml of TCA 20% (three chloroacetic acid), and 0.4 ml of 1.0 M HCl (HCl was added in order to prevent oxidation of polyphenols during extraction and assay processes). The mixture was stirred in a vortex for several seconds and was bathed in the bath for 20 minutes. The mixture then underwent a 20-minute centrifugation for 3500 g (Heraeus Labofuge 200 centrifuge), with the aim of depositing all the solids and receiving a clear solution.

An aliquot of 0.5 ml from the extract fluid was mixed with 1.25 ml of the Folin–Ciocalteu reagent (FCR) and 2.0 ml saturated solution of sodium carbonate. This solution was vortexed for 15 seconds and was kept in the dark for 30 minutes for color development. Then, the solution was centrifuged (5300 rpm) for 10 minutes until it was transparent. The supernatant was collected, and the solution absorbance was measured by using a UV-Vis spectrophotometer (Jasco V-730 Spectrophotometer) at a wavelength of 760 nm. The total amount of polyphenols was expressed as gallic acid equivalents (GAE), based on the calibration curve.

2.4. Lipid Peroxidation Measurement. Lipid peroxidation was determined by measuring the oxidation product of lipids, malondialdehyde (MDA). This molecule is well known as a

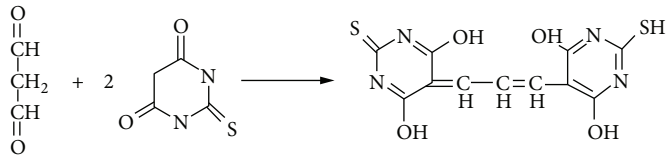

Figure 1: Polyphenol levels in colostrum of healthy and preeclamptic women. The results are shown in mM equivalents of gallic acid. Each column represents mean ± standard deviation.

biomarker and serves as a reliable indirect measure of lipid oxidation. MDA levels were measured spectroscopically by the thiobarbituric acid reactive substances (TBARS), according to a procedure already published by Halliwell and Gutteridge [15] with minor modifications. The assay was based on the thiobarbituric acid (TBA) reaction, a reaction between oxidized lipids and solution of 2-thiobarbituric acid under acidic conditions to yield a pink chromogen with a maximum absorbance at 532 nm (Figure 1) [15]. An aliquot of 1.0 ml of the colostrum slurry (1 ml) was transferred to a 15 ml tube followed by successive additions of 0.67% TBA in 20% TCA and 0.8% BHT in ethanol. The mixture was then homogenized and centrifuged at 5300 rpm for 10 min and incubated in a 70°C water bath for 20 min. Samples were subsequently cooled under tap water for 5 min and centrifuged for 10 min to separate flocculent material. The color produced by the chemical reaction was read at 532 nm against a blank reaction mixture, and the amount of MDA formed was determined by using the molar extinction coefficient $\varepsilon(530\,\text{nm}) = 1.56 \times 10^{-5}\,\text{cm nmol}^{-1}$ [16, 17]. Two assays were carried out for the samples: the Folin–Ciocalteu assay for total polyphenols and the TBARS assay for lipid oxidation measured by determining MDA levels (Figure 2). All results are average of three repetitions.

2.5. Statistical Analysis. All statistical analyses were performed using the SPSS 11.0 program for Windows. Values of variables were expressed as mean ± SD.

3. Results and Discussion

Table 1 presents the characteristics of the study and control group women. In the study group, 14 women were diagnosed with severe preeclampsia and 4 women with mild preeclampsia. Thirteen women were treated with magnesium sulfate.

Polyphenol concentrations are shown in Figure 1. Polyphenol concentrations were significantly higher (about 33% higher, $p = 0.00042$) in colostrum of preeclamptic women in comparison to healthy women. This result surprised us, given the fact that we expected the opposite results. Preeclampsia is known to be a clinical syndrome with a mechanism of oxidative stress. Therefore, it was likely that polyphenol levels would be higher in healthy women's colostrum than in preeclamptic women's colostrum. In the study group, no difference was found in polyphenol levels between women treated and not treated with magnesium sulfate.

Lipid peroxidation levels are shown in Figure 3. The results are shown as the concentration of MDA, which is a measure of lipid oxidation. Significantly lower levels of lipid oxidation were found in the colostrum of women with

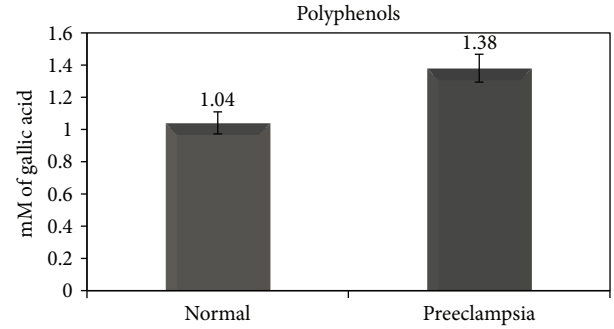

Figure 2: A chemical reaction between MDA and TBA to yield a pink chromogen [9].

preeclampsia compared with healthy women (about 20%, $p = 0.03$). In the study group, no difference was found in polyphenol levels between women treated and not treated with magnesium sulfate.

Again, this result surprised us; however, it works well with the fact that levels of polyphenols, which act as antioxidants, were higher in the colostrum of the preeclamptic population. We assumed that high levels of polyphenols increase the resistance of the milk to the oxidative stress, so there is a reduction in the level of lipid oxidation.

The results indicated higher levels of polyphenols in the colostrum of women that suffers from preeclampsia and lower levels of lipid oxidation. These results appear to be in contradiction to the fact that the mechanism of preeclampsia involves a state of oxidative stress originating from the placenta. At the same time, the explanation for the special phenomenon may be derived from the compensation mechanism in the mother. During the onset of the disease, breast milk may be enriched with polyphenols more in preeclamptic women than in healthy women. This seems to have an evolutionary advantage in protecting the fetus exposed to oxidative damage during labor. The fetus that has suffered from oxidative stress at birth can overcome the damage or prevent further damage by immediate diet of colostrum rich in polyphenols. In order to show whether lipid oxidation levels were correlated with colostrum and polyphenol levels, we combined all the results of the study into one group and examined whether there was an inverse correlation between MDA and polyphenol levels in colostrum. We therefore drew a scatter graph of MDA levels depending on the levels of polyphenols in colostrum. The Pearson correlation coefficient was determined for the regression line and the significance level of the adjustment coefficient. The results are shown in Figure 4.

Table 1: Characteristics of women.

	Study group ($n = 18$)	Control group ($n = 22$)
Age	29 ± 7	30 ± 6
Regular nutrition	22	22
Gestational age (w)	36 ± 4	38 ± 2
Cigarette smoking	None	None
Magnesium sulfate treatment	13 (72%)	None
Spontaneous vaginal delivery	4 (22%)	16 (78%)
Cesarean section	14 (73%)	6 (27%)
Colostrum age (d)	5 ± 1	4 ± 1

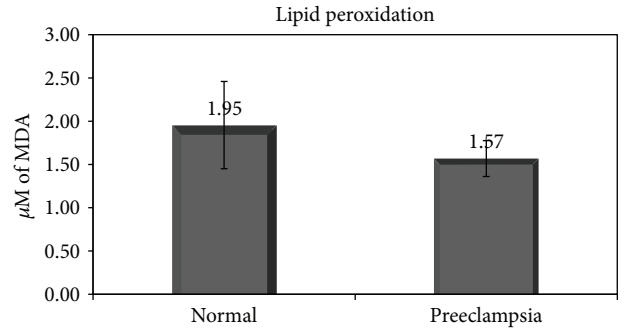

Figure 3: Levels of lipid oxidation in the colostrum of healthy and preeclamptic women. Results are shown in μM of MDA. Results are shown as mean ± standard deviation.

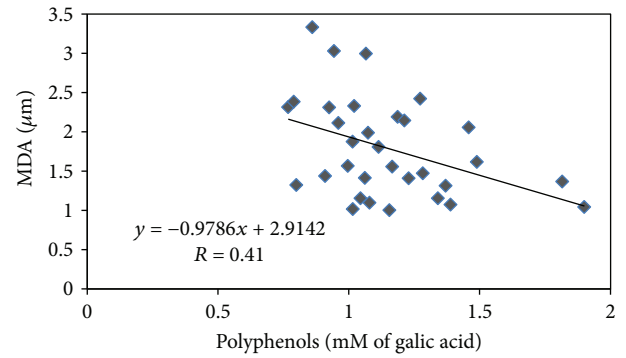

Figure 4: Pearson correlation between the level of lipid oxidation (MDA) and polyphenols in colostrum of healthy women and women with preeclampsia.

A Pearson correlation of -0.41 was obtained between the level of lipid oxidation and polyphenols in colostrum with significance ($p = 0.02$). This means that the higher the levels of polyphenols, the lower the MDA levels. The value of the Pearson coefficient, -0.41, indicates that at a 98% significance level, polyphenols contribute about 40% to the prevention of oxidation of lipids in colostrum. It is likely that other factors, such as activity of antioxidant enzymes and genetic factors, also are involved and influence the process. We analyzed the results according to the ethnicities. No differences were found between the ethnicities. In the preeclampsia group, 11 had CS compared with 5 in the control group. When we analyzed the results within the groups with/without CS, we found that in the control group, a higher level (13%) of MDA was measured in the CS delivery compared with vaginal birth, and a higher level of polyphenols in the vaginal birth compared with CS. No difference was found in the preeclampsia group between those with and without CS. These results support our thesis even more although statistical significance was not reached. Only minority of women had epidural anesthesia: one in the control group and 3 in the preeclampsia group. This did not affect the results.

4. Conclusions

This work has interesting and important conclusions. The colostrum of women with preeclampsia was found to be richer in polyphenols than that colostrum of healthy women. Levels of lipid oxidation were found to be lower in the preeclampsia group. High levels of colostrum polyphenols appear to affect the decrease in lipid oxidation. All differences are statistically significant. The main conclusion is that the woman's body apparently has a compensation mechanism that protects the newborn from the stress processes the mother experiences. These results are consistent with studies showing an increase in the levels of components in breast milk that are supposed to provide protection for the baby in the group of women with preeclampsia [13, 14]. It was shown that milk LCPUFAs and neurotrophins are altered in preeclampsia. LCPUFA could plausibly influence the growth especially in children born to mothers with preeclampsia (18). Preeclampsia may affect milk cytokine balance and offer an immunological signal for the host defense in high-risk neonates [14].

Our recommendation following this study is to encourage women who have experienced preeclampsia to breastfeed their children in the first days of life. We believe this is very important in protecting their newborn.

Our study has potential limitations mainly due to its sample size. In addition, there are more methods to measure polyphenols. We think that similar results to those of other/-different methods could support our study results.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

The Role of Oxidative Stress in the Pathomechanism of Congenital Malformations

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Congenital anomalies are significant causes of mortality and morbidity in infancy and childhood. Embryogenesis requires specific signaling pathways to regulate cell proliferation and differentiation. These signaling pathways are sensitive to endogenous and exogenous agents able to produce several structural changes of the developing fetus. Oxidative stress, due to an imbalance between the production of reactive oxygen species and antioxidant defenses, disrupts signaling pathways with a causative role in birth defects. This review provides a basis for understanding the role of oxidative stress in the pathomechanism of congenital malformations, discussing the mechanisms related to some congenital malformations. New insights in the knowledge of pathomechanism of oxidative stress-related congenital malformations, according to experimental and human studies, represent the basis of possible clinical applications in screening, prevention, and therapies.

1. Introduction

Embryogenesis represents a complex process requiring temporal and spatial regulatory mechanisms [1]. These mechanisms have evolved to be particularly resilient to stressor factors, but experimental studies have shown that embryonic stages are very sensitive to internal or external stressors because of reduced protecting mechanisms [2].

Environmental drugs, chemicals, and physical agents can produce congenital malformations and reproductive effects. The most common known cause is genetic, but the largest group, unfortunately, is unknown.

It is important to remember that a teratogenic exposure includes not only the agent but also the dose and the time in pregnancy when the exposure has to occur. The dose is a crucial component in determining the risk, since those teratogenic agents follow a toxicologic dose-response curve [3].

Known agents that have been demonstrated to result in malformations cannot produce every type of malformation. So, it is easier to exclude an agent as a cause of birth defects than to conclude definitively that it was responsible for birth defects [3].

Oxidation–reduction (redox) homeostasis, like pH control, is central to life. Redox processes pervade practically all fundamental processes of life from bioenergetics to metabolism and life functions [4]. Biological redox reactions are manifold and organized according to the principles of the redox code [5].

Oxidative stress is an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage [6]. Oxidative stress is two sided; whereas excessive oxidant challenge causes damage to biomolecules, maintenance of a physiological level of oxidant challenge, termed oxidative

eustress, is essential for governing life processes through redox signaling [4].

Biological redox equilibria do not denote, as a matter of fact, true thermodynamic equilibria but instead are "nonequilibria" as defined by steady state [7]. Important deviations from the set point in metabolic steady states may ultimately cause damage to biomolecules and can modulate, and even disrupt, physiological redox signaling.

Embryonic development requires specific signaling events that regulate cell proliferation and differentiation to occur at the correct place and the correct time in order to build a healthy embryo. Signaling pathways are sensitive to perturbations of the endogenous redox state and are also susceptible to modulation by reactive species and antioxidant defenses, contributing to a spectrum of passive versus active effects that can affect redox signaling and redox stress [8].

Redox signaling plays a pivotal role in developmental processes, and it is largely regulated during embryogenesis. Disruption of redox signaling pathway alters the control of intracellular redox potential and causes redox stress through the generation of reactive oxygen species (ROS) [4]. These disruptions can include altered cell fate decisions that can lead to structural and functional changes in developing animals, including in specific tissues [9]. ROS and oxidative stress act as teratogenic agents, leading, during embryogenesis, to several structural changes in the developing fetus [8].

In addition to ROS, further important reactive species have notable impacts on redox biology and, consequently, on oxidative stress: reactive nitrogen species (RNS) [10], reactive sulfur species (RSS) [11], reactive carbonyl species (RCS), and reactive selenium species (RSeS) [12].

Enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) are important in scavenging ROS and have been shown to increase 150% during the last 15% of gestation. There are three forms of SOD that have been identified: copper-zinc superoxide dismutase (Cu/ZnSOD) that is present primarily in the cytoplasm, manganese superoxide dismutase (MnSOD) in the mitochondria, and extracellular superoxide dismutase (EC-SOD) located in the extracellular spaces in adults but primarily intracellular in newborns. The only known function of SOD is to convert extremely reactive superoxide radicals to hydrogen peroxide and water. Catalase, GPx, and glutathione reductase then convert hydrogen peroxide to water [13].

Antioxidant enzyme expression generally increases in most fetal compartments throughout the progression of pregnancy. Qanungo and Mukherjea found that SOD, catalase, GPx, and glutathione reductase activities increased with gestational age, as evidence of lipid peroxidation decreased in human placental and fetal tissues [14].

Development of the antioxidant system during fetal life must also include redox signaling in the maintenance of pregnancy through uterine-placental-fetal interactions [15].

There is evidence of regulation of antioxidant enzymes in the context of local nitric oxide (NO) generation via nitric oxide syntheses and downstream NO-dependent signaling in the placenta, critically important to normal vascular development.

In this review, we present data concerning redox signaling in developmental processes and discuss the role of oxidative stress during pregnancy and embryogenesis. Models of congenital malformations in which redox modulations affect the development and function of the system are also shown.

2. Redox Signaling and Oxidative Stress during Embryonic Development

Intracellular ROS are important factors in signaling mechanism, as they modulate physiological processes during embryogenesis. Besides, cellular proliferation, differentiation, and apoptosis are often driven by specific redox signals.

Intracellular superoxide (O_2-) is mainly produced by the oxidation of NADPH by NAPH oxidase enzymes (NOXs) or by electron leak from aerobic respiration in the mitochondria. Superoxide is then quickly converted into hydrogen peroxide (H_2O_2) by superoxide dismutases (SODs). H_2O_2 may either oxidize cysteine residues on proteins to initiate redox biology, or it may be converted to H_2O by cellular antioxidant proteins, such as peroxiredoxins (PRx), glutathione peroxidase (GPx), and catalase (CAT). When H_2O_2 levels raise significantly, hydroxyl radicals (OH) form via reactions with metal cations (Fe^{2+}) and irreversibly damage cellular macromolecules [5].

Hence, the homeostasis of intracellular oxidizing and reducing equivalents is modulated through a fine balance between antioxidant systems, enzymes, and metabolic processes to permit the normal cellular function that occurs when cell signaling is maintained and cellular viability is preserved [6].

Under normal physiological conditions, ROS are quickly destroyed by the antioxidant defense system. Free radical-mediated cellular damage may occur in case of genetic deficiency of free radical scavenging enzyme activity. An imbalance between diminished host antioxidant defenses and increased formation of free radicals (FRs) causes oxidative stress.

An increased production of ROS during organogenesis, period in which cells continue to differentiate, disrupts critical signaling events causing structural abnormalities, loss of cellular function, or spontaneous abortion of the developing fetus [7].

Different conditions may produce abundant ROS in human tissues, resulting in a state of oxidative stress for both the mother and the developing fetus. Although the relationship between oxidative stress and congenital malformations is not clear and need further investigations, experimental studies in animal models have shown that oxidative stress might play a significant causal role in birth defects.

For instance, Long and colleagues investigated the relationship between the toxicological effects of bacterial component LPS via oxidative stress and pulmonary dysplasia in chick embryos. The FGF and Wnt signaling pathways are considered to control lung development. GATA binding protein 6 (GATA-6) is a member of the zinc finger GATA protein family that presumably plays a key role in maintaining the balance between the proliferation and differentiation

of pulmonary epithelial progenitor cells during lung development through modulating Wnt signaling. Long and colleagues demonstrated that LPS could induce the oxidative stress, which subsequently led to altered embryonic lung development. Specifically, LPS induced an intracellular ROS production enhancement, which was partially blunted by the addition of vitamin C to the culture medium. LPS significantly inhibited GATA-6 expression. However, GATA-6 was partially restored by vitamin C. Moreover, LPS induced downregulation of SP-C, ABCA3, and GATA-6 expressions, which again could be restored by vitamin C [16].

Over the course of development, the delicate balance between oxidants and antioxidants can be disrupted by various factors (e.g., thalidomide, phenytoin, ethanol, and maternal diabetes) that induce ROS production and lead to oxidative stress. Many investigators have evaluated the effects of antioxidants on embryonic development. In general, antioxidants reduce the abundance of highly reactive ROS by becoming radicalized themselves. The most important, glutathione, exists in a couple of its oxidized (GSSG) and reduced (GSH) forms. Another significant group of antioxidants are selenium and selenoproteins, such as thioredoxin, GSH peroxidase (GPX4), and selenoprotein W. Lipoic acid is a potent natural antioxidant. Mice deficient in lipoic acid are retarded in their development and die early with a lack of organization and smaller size. The heterozygotes have significantly reduced erythrocyte GSH levels and lower antioxidant capacity.

The enzyme G6PD is also very important to oxidative stress. It is a developmentally critical enzyme that protects the embryo from endogenous and xenobiotic-initiated oxidative stress and DNA damage. In fact, G6PD-deficient dams have higher embryonic oxidation and more fetal death and birth defects than their wild-type counterparts.

Other antioxidants, including vitamin C, are vital to the fetoplacental unit, so that in cryopreserved embryos, addition of ascorbate reduced the levels of hydrogen peroxide, increased the rate of metabolism, and enhanced inner cell mass development [17].

A better understanding of the mechanisms behind the relation between oxidative stress and congenital malformations may be important from both diagnostic and therapeutic perspective, providing both early prenatal diagnostic tools and new possible preventive treatments with antioxidant administration during pregnancy to reduce any oxidative damage of abundant ROS during organogenesis.

Oxidative stability can be assessed using different markers such as antioxidant enzymes: glutathione peroxidase (PxG), superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), vitamin C, vitamin A, vitamin E, glutathione (GSH), or by determining the total antioxidant capacity (TAC) and nitrite levels [8].

Oxidative stress may also affect the levels of PAPP-A and β-HCG, already used to assess the risk of chromosomal aberration in the first trimester [9].

3. Oxidative Stress and Down Syndrome

Plentiful evidence both from in vivo and in vitro studies and animal models have suggested that the pathogenesis of Down syndrome (DS), due to complete or partial trisomy of chromosome 21, might be linked to the effects of oxidative stress on early embryonic development [18].

Trisomy of chromosome 21 causes deregulation of gene/protein expression either in dosage-sensitive genes (gene dosage hypothesis) or in several other genes (amplified developmental instability hypothesis) [19]. Furthermore, additional environmental factors—such as increased production of ROS—could play a central role in determining phenotype severity and the wide clinical variability observed in DS patients [20].

Previous studies have been supposed that a chronic oxidative injury in the brain would act as a risk factor for abnormal brain development, the higher incidence of hyperactivity with attention deficits and Alzheimer disease clinical features of DS patients [21, 22].

Genes located on chromosome 21, overexpressed due to triplication of the chromosome, include copper-zinc superoxide dismutase (SOD1), amyloid precursor protein (APP), transcription repressor BACH1 genes and other several genes with a role in ROS metabolism [23].

SOD1 gene encodes for the enzyme catalyzing the conversion of superoxide anion ($O_2^{\cdot}-$) into the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2) converted into water (H_2O) and molecular oxygen (O_2) by catalase (CAT) and glutathione peroxidase (GPX). The triplication of chromosome 21 causes an excessive activity of SOD1 and an altered ratio of SOD-1/CAT and GPX, resulting in the accumulation of endogenous H_2O_2 and/or its conversion products (hydroxyl radical) with cellular oxidative damage [24]. Interestingly, in several cells and tissues of DS patients, including erythrocytes, B- and T-lymphocytes, and fibroblasts, SOD1 levels are 50% higher than normal, and in all DS tissues there was an altered SOD1/GPX activity ratio [25]. A decreased expression of peroxiredoxin 2, an antioxidant enzyme that detoxifies hydrogen peroxide, was also detected in DS fetal brain [26]. These conditions make neurons of DS patients more sensitive to ROS attack and prone to apoptosis and degeneration [27].

It has also been reported both an increase of intracellular ROS and elevated levels of lipid peroxidation in primary human DS cortical cultures established from cerebral cortex of 16–19 weeks gestation. These evidences suggest that increased generation of ROS in fetal DS neurons leading to neuronal apoptosis may contribute to abnormal brain development and mental retardation and predispose to the early onset of Alzheimer's disease in DS as well [28].

Moreover, the analysis of oxidative stress biomarkers including enzymatic antioxidant defenses (CAT, SOD, and GPX) and oxidative damage antioxidants (protein carbonyls levels and lipoperoxidation), all measured spectrophotometrically in whole blood of 20 DS patients and 18 healthy controls, showed an increase in the SOD and CAT activities and a decrease in protein carbonyls levels in DS individuals, revealing a systemic prooxidant status in the blood of DS patients. Finally, in a cross-sectional study, total SOD activity in plasma from 36 DS children measured by spectrophotometric methods was found increased when compared with 40 healthy controls [29].

Trisomy 21 also causes overexpression of BACH1 gene, a basic leucine zipper protein belonging to the cap'n'collar (CNC) family. BACH1 is a transcription repressor that binds the antioxidant response elements of DNA (AREs) and suppresses the expression of specific genes/proteins controlled by ARE such as quinone oxidoreductase-1 (NQO1), glutathione S-transferase (GST), glutamate-cysteine ligase (GCL), and heme oxygenase-1 (HO-1). In oxidative stress conditions, the function of BACH1 is suppressed thus promoting the expression of these genes involved in the cell stress response. It was supposed that BACH1 overexpression might promote oxidative stress blocking the expression of oxidative stress-responsive and antioxidant genes. Increased total BACH1 protein levels were found into frontal cortex tissue from 16 DS individuals coupled with reduced induction of brain HO-1 compared to healthy individuals, suggesting that BACH1 overexpression in DS leads to the repression of HO-1 transcription and may contribute to the increased OS found in DS [30].

In addition, the overexpression of the amyloid precursor protein (APP) gene, located on chromosome 21, in DS patients causes an increased production of amyloid beta-peptide (Aβ) that is considered to be the most important pathogenic molecule in Alzheimer's disease representing the core protein of neuritic plaques. Aβ accumulates in the brains of DS individuals as early as 8–12 years of age, and this accumulation increases during the lifespan resulting in Alzheimer's disease-like neuropathology found in all DS individuals over 40 years of age. Soluble forms of Aβ generated from APP commonly end at C-terminal residue 40 (Aβ40) or 42 (Aβ42). Plasma concentrations of both Aβ40 and Aβ42, quantitated by sandwich ELISA from 35 DS children and adolescents, were reported significantly higher in DS patients than in controls and the ratio of Aβ42/Aβ40 was lower in DS than in controls [31]. This Aβ-peptide overexpression leads to accumulation into neuritic plaque inducing neuronal loss and cognitive dysfunction and could be associated with ROS production and oxidative stress [32].

Moreover, the overexpression of APP may also induce mitochondrial dysfunction independently from aberrant Aβ deposition, thus aggravating oxidative stress conditions [33].

Furthermore, there is another candidate gene for oxidative stress in DS patients encoding for the enzyme carbonyl reductase (CBR) that normally detoxifies the cytotoxic metabolic intermediates carbonyls catalyzing the reduction of free carbonyls compounds to their corresponding alcohols. Carbonyls are cytotoxic metabolic intermediates that are detoxified by either oxidation catalyzed by aldehyde dehydrogenase (ALDH) or by reduction to alcohols by CBR and/or alcohol dehydrogenase (ADH). Of note, increased levels of CBR protein have been shown in different brain regions of DS patients [34]. Thus, increased levels of CBR could be considered as a marker of oxidative stress, due to its role in detoxification of carbonyls produced by oxidative stress-dependent increases in SOD1 activity.

A systemic prooxidant status in DS individuals has been confirmed in various studies that demonstrated an increased activity of some important antioxidant enzymes (SOD1, CAT, and GR) together with decreased glutathione (GSH) levels in DS whole blood and higher levels of biomarkers of oxidative damage, such as protein carbonyls, malondialdehyde (MDA), allantoin, or 8-hydroxydeoxyguanosine than in controls [35].

Finally, the prooxidant condition in DS patients may be linked to reduced activity of complex I in the respiratory electron transport chain in the mitochondria associated with an increase in cellular ROS [36]. The oligonucleotide microarrays analysis of the expression profile of several genes located on chromosome 21 in 10 samples from cardiac tissue obtained from DS fetuses at 18–22 weeks of gestation after therapeutic abortion revealed a downregulation of genes encoding mitochondrial enzymes and upregulation of genes encoding extracellular matrix proteins. These results show that dosage-dependent upregulation of chromosome 21 genes alters the function of genes involved in mitochondrial function as well as the extracellular matrix organization of the fetal heart of DS patients [37].

In an effort to better understand the role of oxidative stress in DS, a set of oxidative biomarkers were evaluated in amniotic fluid collected from ten women undergoing amniocentesis and carrying confirmed DS fetuses compared with ten women carrying normal fetuses in a retrospective matched case control study [38]. Increased levels of circulating oxidative stress biomarkers were found. Particularly, protein carbonyls and HNE-protein adducts, both evaluated by slot-blot analysis, were found significantly increased in AF from women carrying DS fetuses, suggesting an improving of protein oxidation and lipid peroxidation pathways even at the fetal stage in DS. Glutathione assay results showed a reduction of total glutathione and an increase of GSSG levels with lower Trx levels in DS AF with respect to controls, confirming a loss of thiol-disulfide reductive systems. Furthermore, three heat shock proteins (HSP 70, heat shock protein 70; Grp 78 glucose regulated protein 78; and HO-1, heme oxygenase 1), acting through a cytoprotective mechanism under oxidative stress conditions, evaluated by western blot experiments, were found to be upregulated in DS AF.

It is clear from these data that DS fetuses are exposed to oxidative stress early in pregnancy with consequent damage of many fetal organs and tissues [39].

In conclusion, it has been suggested that trisomy of chromosome 21 causes stress oxidative conditions and oxidative injury early in embryogenesis altering gene/protein expression and particularly inducing overexpression of SOD1 and also reduction of antioxidant enzymes. Moreover, the overproduction of Aβ also affects redox imbalance and could exacerbate oxidative damage into the brain.

Table 1 summarizes the evidence discussed above.

Based on these findings, the administration of antioxidant nutrients could have a role in ameliorating the clinical pattern of DS patients. In previous studies on the effects of antioxidant elements, controversial results were obtained. In a 2-year randomized, double-blind, and placebo-controlled trial with daily oral antioxidant supplementation in DS patients and dementia (900 IU of alpha-tocopherol, 200 mg of ascorbic acid, and 600 mg of alpha-lipoic acid), it was demonstrated that the supplementation was safe

Table 1: Summary of the markers investigating in relation to oxidative damage in Down syndrome.

Marker	DS cell/tissue	Level
SOD1	Erythrocytes	Increased
	B- and T-lymphocytes	Increased
	Fibroblasts	Increased
	Whole blood	Increased activity
Peroxiredoxin 2	Fetal brain	Decreased
CAT	Whole blood	Increased activity
Protein carbonyls	Whole blood	Increased
	Amniotic fluid	Increased
Total Glutathione GSSG	Amniotic fluid	Increased
Trx		Increased
		Decreased
ROS	Primary human cortical cell cultures	Increased
Lipid peroxidation	Primary human cortical cell cultures	Increased
BACH1 protein	Frontal cortex tissue	Increased
Aβ40 and Aβ42	Plasma	Increased
CRB	Brain	Increased
GSH	Whole blood	Decreased
HNE-protein adducts	Amniotic fluid	Increased
HSP 70 Grp 78 HO-1	Amniotic fluid	Increased

and well tolerated but not associated with any stabilization or improvement in the cognitive function [40].

Further studies are needed in order to elucidate the relationship between oxidative stress and DS clinical expression to identify clinical biomarkers of early oxidative stress and damage and to find any possible therapeutic agents.

4. Oxidative Stress and Heart Malformation

The incidence of congenital heart defects (CHDs) varies from 4/1000 to 50/1000 live births [41].

The embryonal heart tube is composed of myocardium and an inner lining of endocardial cells separated by an extensive extracellular matrix the so-called cardiac jelly. The formation of cardiac cushions is a complex event under the direction of specific signaling pathways.

Nowadays, despite there are some progresses in understanding the genetics of heart defects, only 15% of CHDs can be attributed to a genetic cause. All other cases result from a complex interaction between genetic susceptibility and environmental factors (maternal cocaine and alcohol intake, cigarette smoking, exposure to industrial chemicals, viral infections, and so on) whose common embryotoxic effect might be related to excessive production of reactive oxygen species and to reduced antioxidant-defense mechanisms [42].

Despite the role of ROS in cardiovascular diseases (CVD) is well documented [43, 44], there are only few reports concerning the role of ROS in children with congenital heart defects (CHD) [45].

In a study by Ercan and colleagues, the relationship between congenital heart diseases and oxidative stress in children with cyanotic and acyanotic congenital heart diseases was investigated. The authors concluded that the oxidant and antioxidant values of the cyanotic patients were significantly higher than the acyanotic and control groups. So, they have speculated that due to the underlying anatomical defect, hypoxia develops and increases both the free oxygen radicals and the antioxidant substances for compensation afterwards [46].

Increased oxidative stress and reduced antioxidant capacity might lead to CHDs, through ROS production, which affect many intra- and intercellular signaling pathways [47, 48].

Furthermore, recent evidences in humans, i.e., mothers of offspring with congenital heart disease, have shown elevated homocysteine level related to low folate and/or vitamin B12 levels thus supporting that folic acid pathway alteration may exert an indirect embryotoxic effect by increasing oxidative stress. The metabolic pathway from homocysteine to glutathione is referred to as the transsulfuration pathway. Approximately 50% of homocysteine generated from methionine is metabolized to cystathionine. This is an irreversible reaction that permanently removes homocysteine from the methionine cycle for the synthesis of cysteine and glutathione. Elevated homocysteine is associated with alterations in the transsulfuration pathway that lead to greater oxidative stress [49]. In a previous publication, evidence of impairment in remethylation of homocysteine was shown by lower methionine and S-adenosylmethionine concentrations and higher S-adenosylhomocysteine concentrations among women with CHD-affected pregnancies [50]. Current findings indicate that the higher homocysteine observed among women with CHD affected pregnancies may extend beyond impairments in remethylation of homocysteine to impairments in the transsulfuration of homocysteine. Specifically, in comparison to controls, cases with CHD-affected pregnancies had significantly lower concentrations of vitamin B-6, GluCys, and GSH and significantly higher concentrations of GSSG [50].

Experimental models have suggested that, in addition to evidence of a direct teratogenic effect, elevated homocysteine may have an indirect embryotoxic effect by increasing oxidative stress through excessive production of reactive oxygen species and by decreasing the glutathione-dependent antioxidant-defense mechanism. Hobbs and colleagues indicate that higher homocysteine observed among women with CHD-affected pregnancies may extend beyond impairments in remethylation of homocysteine to impairments in the transsulfuration of homocysteine. Specifically, in comparison to controls, cases with CHD-affected pregnancies had significantly lower concentrations of vitamin B-6, glutamylcysteine (GluCy), and reduced glutathione (GSH) and significantly higher concentrations of oxidized glutathione (GSSG).

Role of oxidative stress for congenital cardiovascular malformations is well studied in maternal diabetes [51]. Congenital heart disease occurs in 5% of infants of diabetic mothers.

In fact, diabetic pregnancy is considered an independent risk factor for major embryonic malformations, and cardiac outflow tract defects are among the most frequent alterations observed in epidemiological studies [52]. The highest relative risk for major cardiovascular defects occurs if the mother has gestational diabetes and develops insulin resistance in the 3rd trimester.

Studies on rats have shown that hyperglycemia in the embryo induces production of reactive oxygen species that, together with a reduced ability of fetal cells to activate antioxidant defense mechanisms, mediate adverse effects on cardiac neural crest migration and cardiac outflow tract septation through proapoptotic signaling [53, 54].

In humans, oxidative stress markers have been evaluated in the cord blood of newborns delivered by mothers with diabetes, suggesting that hyperglycemia induces oxidative stress [55]. However, glucose itself is not a mutagen; instead, it may exert a teratogenic effect via a signaling pathway regulating insulin sensitivity. Insulin sensitivity is thought to be involved in the pathophysiology of both type 1 and type 2 diabetes mellitus and insulin, and related signaling pathways are also key mediators of embryogenesis and early development [56].

Glucose may also affect gene expression in embryonic development via epigenetic changes (histone acetylation and microRNA expression) [57].

The alternative that offspring CHD reflects maternally inherited genetic or epigenetic variations that confer risk of both diabetes mellitus and cardiac abnormalities is less likely, because the risk of maternal diabetes mellitus subsequent to birth of a child with CHD was only modestly increased. Conotruncal defect risk increased in the offspring of diabetic women consistent with experimental study findings that hyperglycemia in early pregnancy affects regulatory gene expression in the embryo, leading to cardiac neural crest cell death and increased CHD risk, particularly for conotruncal and outflow tract abnormalities.

Detailed mechanistic studies will be required to define the role of glucose sensitivity in cells from the neural crest and anterior second heart field during cardiac development. Maternal diabetes mellitus was also associated with the entire spectrum of CHD phenotypes. The nonspecific nature of the association suggests that hyperglycemia in early pregnancy may not only influence specific sequences in cardiac development but affects cardiac development in general or exerts its detrimental effect before formation of the primitive heart tube, with subsequent early and late consequences for fetal cardiac development.

Studies in rats have shown that oxidative stress during pregnancy can be reduced by using vitamin E and Vitamin C as antioxidants [58, 59], thereby supporting that ROS are involved in the embryonic dysmorphogenesis of diabetic pregnancy.

Furthermore, recent evidences in humans, i.e., mothers of offspring with congenital heart disease, have shown elevated homocysteine level related to low folate and/or vitamin B12 levels [50, 60], thus supporting that folic acid pathway alteration may exert an indirect embryotoxic effect by increasing oxidative stress [61].

Table 2: Factors that favour or prevent CHD via oxidative stress.

Favouring	Preventing
(i) Maternal diabetes	(i) Vitamin E
(ii) Hyperhomocysteine	(ii) Vitamin C
	(iii) Folic acid
	(iv) Vitamin B12

Despite some human studies have shown that women using multivitamin supplements and folic acid during the periconceptional period had a lower risk of having babies with congenital heart defects, there are still concerns about the role of multivitamin supplements in reducing embryotoxic effect and risk for CHD in humans [62, 63].

Recent studies have also shown that ROS overproduction and/or imbalance in the antioxidant system could lead to pulmonary hypertension in cases of CHD associated with increased pulmonary blood flow in lamb [64] and rodent experimental model of congenital diaphragmatic hernia [65] through a disordered process of vascular remodeling leading to smooth muscle cell hyperplasia, hypercontractility, and endothelial dysfunction [66].

Further studies are needed to identify the key factors in the development of CHDs in order to develop and implement effective primary prevention program with preconceptional screening and development of nutritional intervention.

Table 2 summarizes the factors that favour or prevent CDH via oxidative stress.

5. Role of Oxidative Stress in Neural Tube Defect

The human brain is particularly vulnerable to the damaging effects of reactive oxygen intermediates due to both its complexity and the long period of development (Figure 1).

Embryonic and fetal brain tissues are especially susceptible to peroxidative injury due to the fact that their membranes are rich in easily oxidable polyunsaturated fatty acid side chains [67].

Several studies indicate that antioxidant enzymes and molecules exhibit extremely low activities in fetal tissues, especially the brain [25, 68]. Cim and colleagues demonstrated higher levels of MDA, indicating an increased oxidant status in amniotic fluid of pregnant women with fetal congenital malformations of the central nervous system [69]. Although this antioxidant defense system is adequate to protect brain development under normal conditions, it is easily overcome by ROS, resulting in neurological and morphological abnormalities of SNC [70].

Holoprosencephaly (HPE) is one of the most common birth defects and is characterized by midline defects of the brain, facial, and oral structures. In humans, it has been estimated that HPE affects 1 in every 5000–10,000 live births and 1 in every 200–250 miscarried fetuses [71]. Many cases of

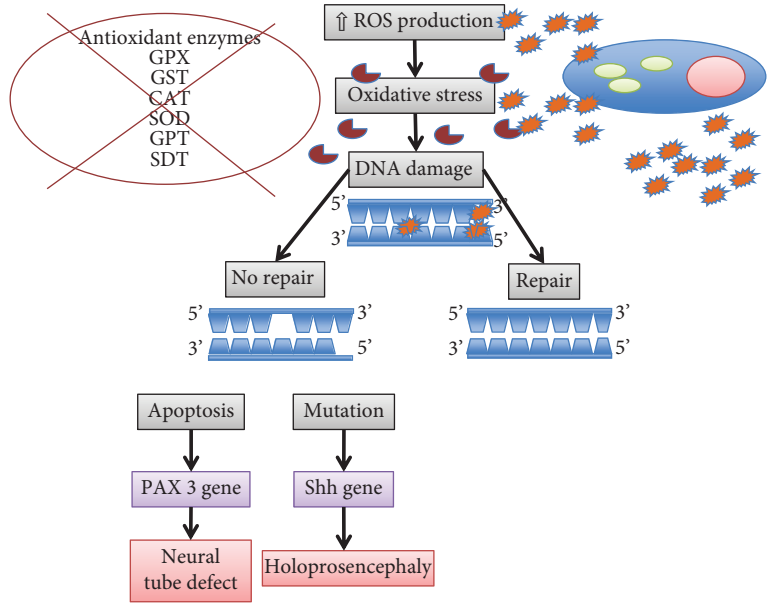

Figure 1: Oxidative stress and CNS malformations.

human HPE occur following fetal alcohol exposure or as a result of maternal diabetes both associated with elevated levels of reactive oxygen species. Studies on the teratogenic mechanism of ethanol-induced HPE in mice have showed that ethanol may impair Sonic hedgehog (Shh) gene expression by activation of protein kinase A (PKA), a potent endogenous negative regulator of Shh signaling during the development of the neural tube [72, 73].

Shh, produced by the axial mesendoderm, prechordal mesoderm (PME), and notochordal plate, acts as a crucial signal in mammalian brain and facial development, and Shh gene alterations are the most frequent causes of autosomal-dominant inherited HPE in humans and Shh2/2 mouse embryos exhibit severe HPE [74–76].

Ethanol-induced activation of PKA in the anterior PME results in a reduction of Shh expression and enhanced apoptosis of anterior PME cells causing the characteristic severe midline defects of HPE.

The inhibition of PME cells apoptosis by antioxidants, i.e., vitamin C and vitamin E, may protect from the teratogenic actions of ethanol.

Neural tube defects (NTDs) are a group of common and devastating congenital malformations that arise early in pregnancy due to the disturbance of normal neural tube closure. NTDs occur in about one in every 1000 established pregnancies worldwide [77], and it is estimated that over 323,000 births were affected with NTDs globally in 2001 [78].

The aetiology of NTD is thought to be heterogeneous, including genetic and environmental factors and their interactions [77, 79]. Factors that have been found to be associated with the risk of NTDs include insufficient folate [80] or multivitamin [81] intake, pregestational and gestational diabetes [82], pesticides [83], and antiepileptic drugs [84]. However, the proportion of NTD cases that can be attributed to known risk factors is lower than one-third [85]. Studies to delineate the mechanism underlying maternal diabetic embryopathy have demonstrated that oxidative stress is a major contributor in NTD formation [86–89]. Excess apoptosis may be one of the mechanisms by which oxidative stress induces malformations. Apoptosis occurs at various developmental stages as a homeostatic mechanism to maintain cell populations in tissues [90]. During the formation of the neural tube, apoptosis appears to be dispensable; however, excessive apoptosis could potentially result in NTDs by causing insufficient cells to be present in the fusing neural folds or by disrupting the physical continuity of the dorsal midline [77, 90]. Growing evidence indicates that oxidative stress can stimulate apoptosis, which may lead to insufficient cell numbers to participate in folding and fusion of neural walls of the neural tube [90, 91].

Moreover, oxidative stress induces DNA damage and defects in DNA repair mechanisms. Single- and double-stranded fractures, base modifications (base participation, rearrangement in some cases), and nucleoside damage may occur in DNA. There may also be crosslinking between DNA and protein depending on oxidative damage [92, 93]. Early embryonic development is vulnerable to oxidative stress because of the immaturity of free radical scavenging mechanisms [69]. The paired box 3 (*Pax3*) gene plays a major role in the development of neuroepithelium of embryos. In the absence of *Pax3*, neural tube defects occur [94]. Oxidative stress occurring before *Pax3* expression leads to an increased risk of neural tube defects and diminished gene expression [95].

Myelomeningocele (MM) is a common congenital malformation that occurs when the embryonic neural tube fails to close properly during early embryogenesis. Common pathogenetic mechanisms for MM include folate deficiency, genetic susceptibility, environmental factors, in utero drug exposure, and biochemical factors [95–100].

Numerous reports have described free radical-mediated congenital defects [86–89]. Kao and colleagues supported

the role of folate in modulating intracellular oxidative stress and suggest an additional mechanism for the etiology of folate deficit-associated MM [101]. Indeed, there is a direct relationship between antioxidant enzymes and the development of MM. GPX, GST, and SOD enzymes are the most important protective systems in humans for neural tube defects. An impaired responsiveness of the antioxidant enzymes, such as CAT, SOD, GPT, and SDT that play an active role in the detoxification of hydrogen peroxidase, has crucial effects in oxygen-induced embryopathy and might result in MM [102, 103].

In addition, SOD is involved in FR-mediated neurological diseases and acting a fundamental role in modulating reactive oxygen species toxicity [104]. In tissues lacking significant catalase activity, detoxification of hydrogen peroxidase becomes critically dependent on GPX. In the study of Graf and colleagues, enzyme activity was abnormal in MM children compared to a control group underlying as deficiencies of enzyme are directly linked to neural tube defects [105].

Arslan and colleagues found that malondialdehyde (MDA), an oxidative damage marker, and a lower activity of erythrocyte carbonic anhydrase, an antioxidant enzyme which regulates the acid-base homeostasis, differ in newborns with MM and in their mothers compared to healthy newborns and their mothers [106].

This study suggests that the finding of low antioxidant enzyme activities in addition to ultrasound and maternal serum alpha fetoprotein may be an index of suspicion of neural tube defect.

In pregnant women with high risk, antioxidant enzymes administration together with folic acid may be an opportunity to reduce the incidence of neural tube defect.

6. Conclusion

Pregnancy is a state of oxidative stress as a consequence of high metabolic activity in the fetoplacental compartment. Fetal tissues are especially sensitive to oxidative damage because of the rapidly growing nature of their cells, which makes them vulnerable to the harmful effects of free radicals.

Despite reactive oxygen species and free radicals, in the presence of a good antioxidant capacity, are important for developing embryos, promoting and controlling cellular fate, and playing a crucial role in normal development through cellular signaling, when overproduced, in the absence of a parallel increase in antioxidative activity, resulted in a wide range of biological toxic effects.

Due to the rapidly growing nature of their cells, fetal tissues are especially sensitive to oxidative damage that lead to lipid, protein, and polysaccharides oxidation and DNA damage with disruption of apoptosis processes that, during organogenesis, are highly needed in an appropriate location and temporal pattern.

Thus, the increase of oxidative stress, together with the impaired antioxidant activity, is clearly related to the induction of fetal malformations.

There are still gaps in our knowledge in the role of oxidative injury in the activation of complex array of genes involved in different biological processes of fetal structure such as inflammation, coagulation, fibrinolysis, cell cycle, cell adhesion, and signal transduction. Future studies addressing the role of oxidative stress in this field are encouraged.

Moreover, there are few published studies evaluating oxidative stress biomarkers and management of oxidative stress with antioxidants therapeutic approaches.

Oxidative stress is widely implicated in failed reproductive performance, including infertility, miscarriage, diabetes-related congenital malformations, and preeclampsia. Poston et al. have focused on the role of free radicals and antioxidant capacity in preeclampsia. By measuring markers of lipid peroxidation and antioxidant capacity, they demonstrated the role of oxidative stress in this disorder [107].

Recent studies suggest that ischemia-reperfusion in the placenta as well as endoplasmic reticulum stress in the placenta may contribute to oxidative stress in trophoblasts. The recognition of oxidative stress in the placenta and the maternal circulation has led to evaluate the potential benefit of prophylactic antioxidant supplementation in women with a known risk of preeclampsia, particularly with an early supplementation with vitamins C and E [108]. However, until now, trials have shown no evidence that these supplements can prevent preeclampsia, but it is important to underline that no RCT has yet addressed prophylaxis over the periconceptual period.

Other potential approaches include the use of supplements in the preconceptual period, selenium supplements, antiperoxynitrite strategies, and statins [109, 110].

In clinical practice, early markers of oxidative stress might reveal that gravidic prophylactic use of antioxidants could help to prevent or at least reduce oxidative stress-related malformations in fetuses. Anyway, maternal antioxidant supplementation during pregnancy is important for protecting newborns against oxidative DNA damage.

Potential therapies for ROS-induced disease include both enzymatic and nonenzymatic antioxidant preparations. Supplementation with enzymatic and/or nonenzymatic antioxidants might have beneficial effects in decreasing injury from excess production of ROS, particularly in disorders such as bronchopulmonary dysplasia, retinopathy of prematurity, periventricular leukomalacia, and necrotizing enterocolitis in preterm newborns who are especially susceptible to ROS-induced damage because of inadequate antioxidant stores at birth, as well as impaired upregulation in response to oxidant stress [13]. Nonenzymatic proteins (transferrin, ferritin, and ceruloplasmin), enzymes (superoxide dismutases, catalase, and glutathione peroxidase), oxidizable molecules (glutathione, vitamins E, A, C, carotenoids, and flavonoids), and trace elements (copper, zinc, and selenium) all play a role in maintaining a delicate balance between ROS production and oxidant damage to tissues and organs [111, 112].

More research is required to determine the beneficial effects of supplemental antioxidant therapy. There are multiple potential therapeutic antioxidants currently under investigation that could benefit newborns, particularly premature infants. For example, one protein under investigation

is Pon3 that was shown in laboratory studies to have antioxidant properties and to be upregulated in rat, sheep, and human cord blood late in gestation [113]. Other clinical trials include supplementation of preterm infants with lactoferrin and cysteine, examination of concentrations of beta-carotene, lutein, and lycopene in preterm infants fed formulas with mixed carotenoids and the effects on the developing eye, early administration of human erythropoietin in very preterm infants, NAC administration to women with intra-amniotic infection and/or inflammation, early enteral administration of vitamin E to extremely premature infants, and multiple trials involving inhaled nitric oxide. The results from these trials may change the way we treat many common neonatal conditions.

Caution must be taken since ROS are critical second messengers in various cell signaling pathways that control normal cellular functions, but strategies that maintain normal antioxidant balance may be beneficial to the newborns. New studies should more extensively investigate the diagnostic and therapeutic value of various oxidative stress biomarkers and antioxidants to reduce oxidative tissue injury to developing newborns.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Impact of Obesity and Hyperglycemia on Placental Mitochondria

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A lipotoxic placental environment is recognized in maternal obesity, with increased inflammation and oxidative stress. These changes might alter mitochondrial function, with excessive production of reactive oxygen species, in a vicious cycle leading to placental dysfunction and impaired pregnancy outcomes. Here, we hypothesize that maternal pregestational body mass index (BMI) and glycemic levels can alter placental mitochondria. We measured mitochondrial DNA (mtDNA, real-time PCR) and morphology (electron microscopy) in placentas of forty-seven singleton pregnancies at elective cesarean section. Thirty-seven women were normoglycemic: twenty-one normal-weight women, NW, and sixteen obese women, OB/GDM(−). Ten obese women had gestational diabetes mellitus, OB/GDM(+). OB/GDM(−) presented higher mtDNA levels versus NW, suggesting increased mitochondrial biogenesis in the normoglycemic obese group. These mitochondria showed similar morphology to NW. On the contrary, in OB/GDM(+), mtDNA was not significantly increased versus NW. Nevertheless, mitochondria showed morphological abnormalities, indicating impaired functionality. The metabolic response of the placenta to impairment in obese pregnancies can possibly vary depending on several parameters, resulting in opposite strains acting when insulin resistance of GDM occurs in the obese environment, characterized by inflammation and oxidative stress. Therefore, mitochondrial alterations represent a feature of obese pregnancies with changes in placental energetics that possibly can affect pregnancy outcomes.

1. Introduction

The placenta is a metabolically active organ with multiple functions connecting the mother and the fetus for a successful outcome of pregnancy [1].

Mitochondrial oxidative phosphorylation and substrate oxidation represent the main energy source for placental function [2]. Therefore, mitochondrial function or dysfunction plays an important role in metabolic health and cellular fate [3]. In the human and rodent placenta, both nutritional and hypoxic stresses can alter mitochondrial function [4–10], with changes in mitochondrial biogenesis, function, and morphology leading to placental dysfunction. Placental alterations can affect fetal metabolism and development possibly leading to higher risk of developing disease in the future adult [11].

In the last decade, obesity has become a global problem [12]. Maternal obesity (MO) is expanding exponentially worldwide to almost epidemic proportions, with an additional 5–10% of pregnant women with diabetes, representing a significant risk factor for adverse pregnancy outcomes [13–17] with both immediate and long-term consequences [11, 18–22]. However, molecular mechanisms underlying programming effects have been only partially identified. Impaired placental transfer and metabolism of energy substrates in maternal obesity and/or diabetes mellitus have been reported [23, 24]. A lipotoxic placental environment is indeed recognized in maternal obesity, with an altered metabolome profile, increased inflammation and oxidative stress, and decreased regulators of angiogenesis [25–28]. This might alter mitochondrial function, resulting in excessive production of reactive oxygen species and oxidative

stress, in a vicious cycle leading to placental dysfunction and impaired pregnancy outcomes.

In this study, we addressed the hypothesis that maternal pregestational body mass index (BMI) and glycemic levels can alter placental mitochondria, by measuring mitochondrial content and morphology in term placentas sampled at elective cesarean section.

2. Materials and Methods

Pregnant women were enrolled in the Unit of Obstetrics and Gynecology of the Luigi Sacco Hospital in Milan.

The study protocol was approved by the local Institutional Review Board (Luigi Sacco Hospital Ethical Committee), and all participants gave their informed consent.

2.1. Population. Only Caucasian women with singleton spontaneous pregnancy and delivering at term by elective cesarean section were included in this study. Cesarean sections before labor were performed for breech presentation, repeated caesarean section, or maternal request. Exclusion criteria were represented by maternal-fetal infections or autoimmune diseases, maternal smoking and drug-alcohol abuse, fetal malformations, chromosomal disorders, preeclampsia, and intrauterine growth restriction.

Forty-seven pregnant women were eligible for the study.

Thirty-seven presented normal glycemia values based on an oral glucose tolerance test (OGTT—75 g) [29]. Among them, twenty-one were within normal weight (NW; $18.5 \leq \text{BMI} < 25\,\text{kg/m}^2$) and sixteen were obese (OB/GDM(−); $\text{BMI} \geq 30\,\text{kg/m}^2$) according to their pregestational BMI [30].

Ten women were diagnosed with gestational diabetes mellitus (GDM) according to the OGTT at 24–28 weeks of gestation, and all of them were obese (OB/GDM(+)). Women with GDM underwent daily checks of glycemia.

All women were given nutritional and lifestyle advice and recommendations on weight gain during pregnancy following IOM guidelines, depending on pregestational BMI [30].

Obese patients had regular specific checkups in a dedicated antenatal clinic, where they received specific dietary indication to support the control of their gestational weight gain and their glycemia levels. No patient needed insulin therapy.

NW women had physiological pregnancies with a normal intrauterine growth and appropriate for gestational age birth weight according to reference ranges for the Italian population [31].

2.2. Data Collection. Maternal medical history, demographic, anthropometric, obstetric, and neonatal data were recorded at recruitment and after cesarean delivery.

Maternal hemoglobin was measured at 34–36 weeks. Maternal fasting glycemia was obtained from the first value of the OGTT performed between 24 and 28 weeks.

2.3. Sample Collection and Processing. Human placentas were collected immediately after elective cesarean section, in the absence of labor. Placentas were weighed after discarding membranes and cord from the disc, and biometric measurements were performed as previously described [18].

After removing the maternal decidua, placental biopsies ($\sim 1\,\text{cm}^3$) were sized from different cotyledons [32] midway between the cord insertion and placental border. Placental villi were then washed in PBS (Dulbecco's phosphate-buffered solution; Euroclone, Milano, Italy) and immediately frozen in liquid nitrogen to be stored at −80°C until mtDNA analysis or alternatively were fixed with 2.5% glutaraldehyde for electron microscopy.

2.3.1. Placenta mtDNA Content. Frozen placental fragments (90 mg) were minced in a TRIzol reagent (Roche Diagnostics, Indianapolis, IN, USA) with a Potter homogenizer. Total DNA was isolated from this mixture with a chemical procedure, following the manufacturer's instructions. DNA concentrations were measured by a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies; Wilmington, DE, USA).

Mitochondrial DNA (mtDNA) content was assessed by real-time PCR, normalizing levels of a mitochondrial gene (cytochrome-β, CYB) to those of a single-copy nuclear gene (RNase-P) ($2^{-\Delta Cq}$ method). Briefly, 30 nanograms of total DNA was analyzed in triplicate with TaqMan assays (CYB: Hs02596867_s1 and RNase-P: 4316844) by 7500 Fast Real-Time PCR (Applied Biosystems, Thermo Fisher Scientific; Carlsbad, CA, USA); Cq values with standard deviation exceeding 0.25 were excluded.

2.3.2. Electron Microscopy. Cells were fixed with 2.5% glutaraldehyde in 100 mM cacodylate buffer pH 7.4 for 1 hour at room temperature. After several washes in cacodylate buffer, cells were postfixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide in 100 mM cacodylate buffer pH 7.4 for 1 hour on ice. After a rinse in dH_2O, samples were en bloc stained in 0.5% uranyl acetate overnight and dehydrated in increasing concentrations of ethanol and finally embedded in Epon. Samples were cured at 60°C in an oven for 48 h. Epon blocks were sectioned using a Leica EM UC7 ultramicrotome (Leica Microsystems, UK). Ultrathin sections (70 nm) were contrasted with 2% uranyl acetate and Sato's lead solutions and observed with a LEO 912AB Zeiss Transmission Electron Microscope (Carl Zeiss, Oberkochen, Germany). Digital micrographs were taken with a $2\text{k} \times 2\text{k}$ bottom-mounted slow-scan ProScan camera (ProScan, Lagerlechfeld, Germany) controlled by the EsivisionPro 3.2 software (Soft Imaging System, Münster, Germany).

2.4. Statistical Analyses. Data are presented as mean ± standard error.

Maternal, fetal, and molecular data were compared among study groups by one-way analysis of variance (ANOVA), having preliminarily verified that no serious statistical violations occurred. Tukey HSD test was then run as a post hoc test.

A two-way between-group ANOVA was conducted to explore the impact of maternal BMI/GDM and fetal sex (independent variables), as individual or joint effect, on placental levels of mtDNA (dependent variable).

Table 1: Maternal, fetal and placental characteristics in the study population.

	NW, $n = 21$	OB/GDM(−), $n = 16$	OB/GDM(+), $n = 10$
Maternal data			
Age, years	35.7 ± 1.02	32.7 ± 1.26	35.7 ± 1.39
Pregestational BMI, kg/m^2	21.5 ± 0.45	34.9 ± 1.17***	35.6 ± 1.46***
Fasting glycemia, mg/dl	81.4 ± 1.72	82.0 ± 1.80	90.4 ± 4.13*
Hb, g/dl	11.4 ± 0.20	10.7 ± 0.34	11.1 ± 0.27
GWG, kg	10.38 ± 0.58	7.94 ± 1.06	8.50 ± 1.57
GWG to IOM advised limits, %	64.9 ± 3.65	95.5 ± 9.18	92.8 ± 17.43
Fetal & placental data at delivery			
Gestational age, weeks	39.2 ± 0.12	39.1 ± 0.07	39.1 ± 0.05
Fetal weight, g	3329.6 ± 63.1	3339.1 ± 87.8	3394.0 ± 126.0
Placental weight, g	460.6 ± 19.2	488.4 ± 22.0	559.0 ± 20.5**
Placental efficiency	7.54 ± 0.41	7.00 ± 0.30	6.11 ± 0.23*
Placental area, cm^2	257.5 ± 15.7	243.7 ± 16.2	259.3 ± 17.7
Placental thickness, cm	1.84 ± 0.12	2.12 ± 0.15	2.25 ± 0.17
Umbilical vein Hb, g/dl	14.0 ± 0.34	14.4 ± 0.48	13.7 ± 0.51

Data are presented as mean ± standard error. Post hoc comparisons using the Tukey HSD test: $^*p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$ versus NW. NW: normal weight; OB/GDM(−): obese without a diagnosis of GDM; OB/GDM(+): obese with GDM; GDM: gestational diabetes mellitus; BMI: body mass index; maternal fasting glycemia: referred to the first value of the oral glucose tolerance test (OGTT); Hb: hemoglobin; GWG: gestational weight gain; IOM: Institute of Medicine; placental efficiency: fetal/placental weight ratio; placental area: (larger diameter) × (smaller diameter) × (π/4).

Chi-square analyses were performed to compare anemia frequencies among groups, using Yates continuity correction.

Correlations describing the strength and direction of the relationships between 2 parameters were assessed using the Pearson product-moment correlation.

All statistical tests were 2-sided, and p values < 0.05 were considered statistically significant. Statistical analysis was performed using SPSS (version 24.00, IBM Statistics; Armonk, NY, USA).

3. Results

3.1. Characteristics of the Study Population. Maternal, fetal and placental data are reported in Table 1.

According to inclusion criteria, pregestational BMI was significantly different among groups ($F(2, 46) = 78.52$, $p \le 0.001$), being higher in the two obese groups compared to NW. Obese women gained on average less weight during pregnancy compared to normal weight, mostly remaining within IOM recommended limits for gestational weight gain during pregnancy of obese women [30]. As expected, maternal fasting glycemia was significantly different among groups ($F(2, 46) = 3.71$, $p = 0.03$), with OB/GDM(+) showing higher levels compared to normoglycemic groups (Tukey HSD test, $p = 0.03$). Hemoglobin levels were lower in the two OB groups compared to NW, though not significant. However, anemia (Hb < 11.0 g/dl) frequency was higher in OB, resulting more than two-fold higher in OB/GDM(−) (56%) and in OB/GDM(+) (50%) than in NW (25%) subjects.

Maternal age, gestational age, and fetal weight did not differ among groups.

There was a statistically significant difference in placental weight ($F(2, 46) = 4.75$, $p = 0.01$). Post hoc comparisons using the Tukey HSD test indicated that the mean score for OB/GDM(+) was significantly higher compared to the NW group ($p = 0.01$).

Placental efficiency (fetal/placental weight ratio) was significantly different among groups ($F(2, 46) = 3.29$, $p = 0.04$), with OB/GDM(+) showing decreased placental efficiency compared to NW ($p = 0.03$).

In our study population, placental efficiency was significantly and positively correlated with maternal Hb ($p = 0.005$, $r = 0.412$, Figure 1) and with gestational age ($p = 0.001$, $r = 0.457$), while it was negatively correlated with placental thickness ($p = 0.03$, $r = -0.336$). Maternal Hb also positively correlated with gestational age ($p = 0.004$, $r = 0.422$) and negatively with placental weight ($p = 0.03$, $r = -0.323$) (data not shown).

Among the analyzed pregnancies, 23 carried male fetuses (15 of NW, 6 of OB/GDM(−), and 2 of OB/GDM(+) mothers) and 24 carried female fetuses (6 of NW, 10 of OB/GDM(−), and 8 of OB/GDM(+) mothers).

3.2. mtDNA Content in Placental Tissue. A one-way between-group analysis of variance was conducted to explore the impact of obesity and GDM on levels of mitochondrial DNA. There was a statistically significant difference among groups in mtDNA levels ($F(2, 46) = 3.03$, $p = 0.49$). Post hoc comparisons using the Tukey HSD test indicated that the mean score for the OB/GDM(−) group was significantly higher compared to NW ($p = 0.047$), while OB/GDM(+) was not (Figure 2).

Figure 3(a) shows the relation between mtDNA levels and pregestational BMI in the study population. There was a significant correlation in patients without a diagnosis of GDM, indicated by the regression line ($p = 0.010$, $r = 0.419$),

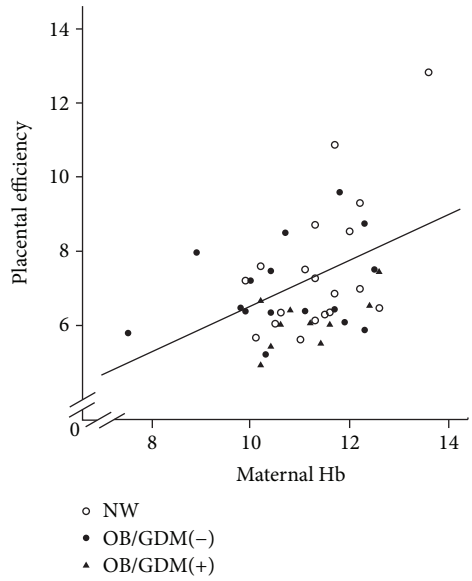


Figure 1: Significant correlation between placental efficiency and maternal hemoglobin ($p = 0.005$, $r = 0.412$). NW: normal-weight women; OB/GDM(−): obese women without a diagnosis of GDM; OB/GDM(+): obese women with GDM.

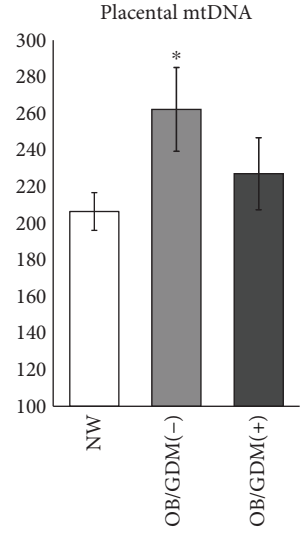


Figure 2: Placental mtDNA levels. $^*p = 0.047$ versus NW, Tukey HSD test. NW: normal-weight women; OB/GDM(−): obese women without a diagnosis of GDM; OB/GDM(+): obese women with GDM.

while no significant correlation was found in OB patients with GDM. mtDNA also negatively correlated with maternal Hb ($p = 0.011$, $r = -0.373$) (Figure 3(b)) and umbilical vein Hb ($p = 0.019$, $r = -0.406$) (Figure 3(c)).

There were no differences in mtDNA levels depending on fetal sex. The two-way between-group analysis of variance showed that the interaction effect between fetal sex and maternal pregestational BMI was not statistically significant ($F(2, 46) = 0.61$, $p = 0.94$) (data not shown).

3.3. Electron Microscopic Analysis of Syncytiotrophoblast. The mitochondrial profiles in the syncytiotrophoblast of both NW and OB/GDM(−) placentas were round or elongated with a very dense matrix and similar structure of the cristae (Figures 4(a) and 4(b)). In contrast, syncytiotrophoblast mitochondria in OB/GDM(+) placentas displayed morphological abnormalities, showing a matrix with very low density and vesicle-like or disrupted cristae, forming an irregular pattern (Figure 4(c)).

4. Discussion

Recently, maternal obesity has been associated with a lipotoxic placental environment, with increased placental lipids, inflammation, and oxidative stress, together with a less efficient fetal/placental ratio and altered metabolome profile [18, 23, 25, 27]. The cellular stress characterizing this maternal environment may adversely affect placental development and function possibly altering fetal growth and development. Indeed, oxidative stress is one of the hallmark responses to intracellular lipid overload. High levels of free fatty acids impact the mitochondrial (mt) membrane structure, causing the release of reactive oxygen species (ROS) that can react with macromolecules and damage intracellular membranes and DNA [33]. These alterations can in turn affect mitochondrial structure and function, in a vicious cycle of mitochondrial abnormalities and ROS formation, possibly representing a key mechanism of placental dysfunction in a disease condition.

Several animal models of MO report mt dysfunctions in pancreatic islets, liver, or skeletal muscle of the offspring [34, 35]. However, maternal obesity and diabetes are not always associated with obvious fetal distress, and the possible placental adaptation may explain it [24].

In this study, we addressed the hypothesis that placental mitochondria in pregnancy can be altered by elevated maternal BMI and/or by metabolic alterations occurring in gestational diabetes mellitus.

We studied placentas at term only delivered by elective cesarean section, in order to avoid possible alterations of mitochondrial content or function due to labor [36]. Obese patients were followed during pregnancy with a specific counseling including nutritional and lifestyle advices. This resulted in lower gestational weight gain compared to normal weight patients, as recommended by IOM guidelines [30]. Our study population was also carefully selected by excluding women carrying further conditions possibly affecting mitochondrial characteristics, such as maternal smoking or drug-alcohol abuse and maternal or fetal pathologies. Obese women with GDM were included, in order to evaluate the additional effect of increased glycemia to placental mitochondrial features.

4.1. mtDNA in Obese Pregnancies without GDM. mtDNA levels are largely recognized as a measure of the mitochondrial content [4, 37, 38]. We found higher levels of mtDNA, accounting for the increased mitochondrial content in placental cells of women with an obese pregestational BMI without a diagnosis of GDM (Figure 2). The morphology of

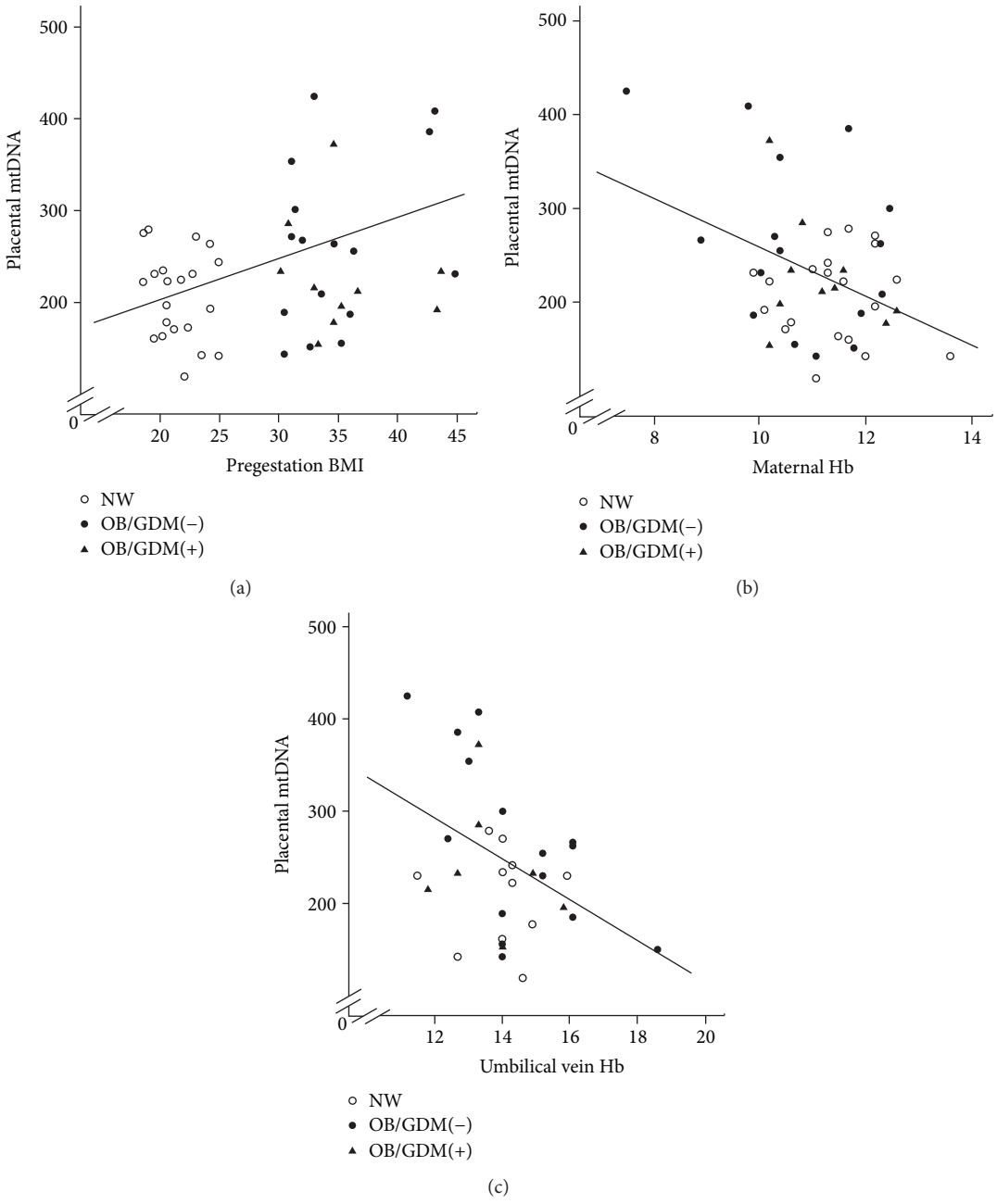
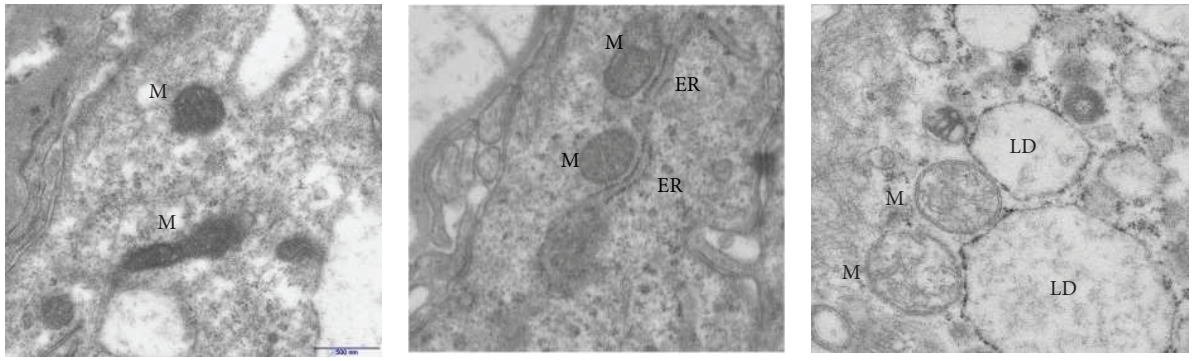


Figure 3: (a) Correlation between placental mtDNA and maternal pregestational BMI. The correlation is significant in patients without a diagnosis of GDM, indicated by the regression line ($p = 0.010$, $r = 0.419$). (b). Correlation between placental mtDNA and maternal hemoglobin ($p = 0.011$, $r = -0.373$). (c). Correlation between placental mtDNA and umbilical vein hemoglobin ($p = 0.019$, $r = -0.406$). NW: normal-weight women; OB/GDM(−): obese women without a diagnosis of GDM; OB/GDM(+): obese women with GDM.

mitochondria in the syncytiotrophoblast of the OB/GDM(−) group was overall similar to the NW group, suggesting no alterations in mitochondrial function (Figures 4(a) and 4(b)). A compensatory increase in mitochondrial biogenesis can be explained by the endocrine stimuli due to high intracellular fatty acid levels and oxidative stress occurring in the lipotoxic environment of obese placentas [9, 34]. Indeed, altered levels of mtDNA as well as the impairment of nutrient transport systems have been reported in previous studies in the placental tissue of different pregnancy pathologies characterized by elevated oxidative stress and inflammation levels, such as intrauterine growth restriction and preeclampsia [4, 5, 39–43]. The positive correlation between placental mtDNA and maternal BMI that was observed in this study supports this hypothesis (Figure 3(a)).

Differently from our study, decreased mtDNA copy number has been previously reported in placentas of obese compared to not-obese women [9, 44]. However, different gene assays and different population criteria were employed in these studies. One of the strengths of the present study is



(a) (b) (c)

Figure 4: Electron microscopy of term placenta villi showing representative sections of syncytiotrophobast cells from NW (a), OB/GDM(−) (b), and OB/GDM(+) (c) term placentas. M: mitochondria; ER: endoplasmic reticulum; LD: lipid droplet. NW: normal-weight women; OB/GDM(−): obese women without a diagnosis of GDM; OB/GDM(+): obese women with GDM.

the careful selection of a very well characterized population of uncomplicated pregnancies. We excluded any maternal or fetal infection or autoimmune disease, maternal smoking or drug-alcohol abuse, fetal malformations, chromosomal disorders, preeclampsia, and intrauterine growth restriction, all of which can affect mitochondrial biogenesis and functionality. Moreover, only Caucasian women were selected, as different mitochondrial DNA haplogroups have been identified and have been suggested to possibly contribute to the genetic component of complex disorders [45–47]. Finally, all women included in this study were counseled with nutritional and lifestyle advice and recommendations on weight gain during pregnancy, and obese patients had regular specific checkups in a dedicated antenatal clinic with specific dietary indication.

Interestingly, in a recent review on mitochondrial features in gestational disorders, Holland and colleagues reported that mitochondrial content has been found to be increased or decreased in the same pregnancy pathology by different studies [48]. These apparent differences within the same pathologies can be explained by the different severity or timing of the insult and the resulting capacity of the tissue to respond. Indeed, although in several diseases, mitochondrial biogenesis is thought to occur as a compensatory mechanism to the cell distress [4, 49–52], on the other hand, the increase of mitochondrial ROS production could damage the mitochondrial DNA and membranes, thus inhibiting the adaptive mitochondrial increase.

Therefore, the different inclusion/exclusion criteria of the studied population, together with different clinical protocols in nutritional and lifestyle advice, can lead to different results regarding the mitochondrial responses. This is also suggested in the recent study reporting increased placental mitochondrial content in early-onset but not late-onset preeclampsia [41].

4.2. mtDNA in Obese Pregnancies with GDM. When analyzing placentas of obese women with GDM, we did not find a significant increase in mtDNA levels (Figure 2). However, placentas of OB/GDM(+) women showed dysfunctional syncytiotrophoblast mitochondria, with morphological abnormalities. In particular, electron microscopy revealed a loss of matrix density and disorganization of inner membrane cristae (Figure 4(c)). Interestingly, also animal models of diabetes showed a reduced number of mitochondria, with abnormal morphology, associated to mitochondrial dysfunction [53]. Notably, large lipid droplets that stock lipids as energy-rich storage compounds were observed. In this context, lipid droplets may support feeble mitochondrial function by supplying fatty acids for mitochondrial β oxidation and protect mitochondria from lipotoxicity [54].

Our results therefore suggest that placental mitochondria of obese women with GDM do not show significant alterations in biogenesis but present altered morphology, indicating an impairment of their function.

The OB/GDM(+) group also presented decreased placental efficiency compared to NW. Indeed, GDM has been associated with impaired placental development showing villous immaturity or alterations in villous branching, as well as impaired placental angiogenesis, villous vasculature, and uteroplacental perfusion [55–57]. Oxidative stress markers have also been reported in GDM placentas [58], possibly affecting the physiology of the placental vasculature and mitochondrial morphology.

Insulin resistance and altered metabolic profiles characterize the diabetic condition. Insulin resistance has been correlated in several tissues with a decrease in mitochondrial function and mitochondrial DNA copy number, a reduction in mitochondrial fusion and increase in their fission and with alterations of mitochondrial size and density. The possible role of epigenetic regulation is emerging for these alterations [59–64]. Indeed, some authors recently hypothesized that insulin resistance acts on the expression of proteins involved in the methylation machinery of both nuclear and mitochondrial DNA, affecting the expression of genes involved in mtDNA replication, thus leading to decreased mitochondrial biogenesis [62, 65, 66]. However, other studies report either no impairment or a compensatory increase of mitochondrial function and oxidative capacity in conditions of insulin resistance [67, 68]. Hence, the relationship between

mitochondria and insulin action is highly complex and there is still much to learn in this area [3].

Similarly to our results, decreased levels of placental mtDNA have been recently reported in diabetic pregnancies [69], together with lower mitochondrial respiratory chain enzyme activities [9]. In our population, different levels of placental mtDNA in obese with or without GDM may be the result of opposite strains. Maternal diabetes has been associated with a decrease of placental mitochondrial levels [9, 69], while the obese environment associated with inflammation and oxidative stress tends to promote a compensatory mitochondrial biogenesis. In the recent study, we reported comparable results in the maternal blood of obese women with or without GDM [52]. Levels of mtDNA in maternal blood may indeed result by the release of placental cell debris in the maternal circulation [70].

In addition, complex changes in the metabolic profile have been shown in obese pregnant women with or without GDM [25, 71]. White et al. recently showed that in addition to the dysregulation of glucose metabolism, GDM obese women compared with non-GDM obese women exhibited exaggerated dyslipidemic profiles prior to the GDM diagnosis, at week 17, when placentation still occurs. This possibly reflects enhanced insulin resistance in peripheral tissues of GDM women and a consequent reduced suppression of lipolysis, affecting lipid metabolism pathways. Increased insulin resistance and higher levels of lipids and lipoproteins have also been shown at mid and late pregnancy in women with GDM compared to normal glucose tolerant patients [72].

Moreover, GDM-obese women showed metabolic patterns consistent with perturbed energy pathways [25, 71]. Particularly, obese women with GDM showed increased levels of acetoacetate (likely secondary to unregulated fatty acid oxidation in mitochondria) and citrate (an early intermediate of the tricarboxylic acid cycle, occurring in mitochondria). These evidences suggest a specific metabolic milieu of GDM compared to non-GDM obese pregnant women, which can differently affect the mitochondrial function in placenta.

4.3. Relation between mtDNA and Maternal/Fetal Hemoglobin. Noteworthy, in our study population, we found a significant negative correlation between placental mtDNA and hemoglobin levels in the maternal and fetal blood (Figures 3(b) and 3(c)). Maternal Hb also significantly correlated with placental efficiency.

Maternal systemic hemoglobin may account for maternal nutritional status. Obese women in this study did not present significantly lower levels of maternal Hb, possibly due to the specific and regular nutritional counseling given to them during pregnancy. However, in our population anemia frequency (Hb < 11.0 g/dl) was higher in both OB subgroups compared to NW. Indeed, obesity induces a chronic, low-grade inflammation with overexpression of C-reactive protein and hepcidin [26, 52] that is negatively correlated with both maternal and cord blood iron status [73]. On the other hand, low maternal Hb may be an index of altered vascular oxygenation that can induce mitochondrial biogenesis, according to the negative correlation we found in our study population.

4.4. Limitations. A sexual dimorphism has been reported in several placental responses to adverse environments [13, 18, 74]. Nevertheless, in our study population, we did not find any interaction effect between the fetal sex and maternal pregestational BMI, suggesting that the BMI influence on mtDNA levels and morphology does not depend on fetal sex. However, a limited number of cases within each subgroup might lead to these results. Therefore, further investigations are needed to explore the possible effect of fetal sex on the mitochondrial content and function in placentas of obese pregnancies with or without GDM.

Although our results on placental mitochondria of OB/GDM(+) pregnancies comply with previous findings, showing lower mitochondrial copy number compared to NW placentas [9, 69], inclusion criteria of the populations analyzed in these studies were different to ours, also including type 1 and type 2 diabetes mellitus, placentas from vaginal deliveries, and different ethnic groups, thus keeping open the need of further studies investigating the consequences of insulin action on placental mitochondria in GDM pregnancies.

Moreover, whether alterations in mtDNA content of OB placental whole tissue are due to alterations in one or more placental cell types still remains to be investigated.

5. Conclusions

The placenta integrates nutritional and endocrine signals and arranges its metabolic phenotype to support pregnancy. The metabolic response of the placenta to impairment depends on the nature, severity, and duration of the environmental adversity [1], and in obese pregnancies, these can possibly vary depending on several parameters like maternal glycemia or the maternal nutritional status and lifestyle. However, mitochondrial alterations are a clear feature of obese pregnancies with changes in placental energetics and consumption of oxidative substrates that possibly can affect fetal delivery of nutrients and O_2 with short- and long-term consequences on the newborn.

Understanding the differences in placental metabolic adaptation to obesity and insulin resistance might open new perspectives for therapeutic future developments [24].

Data Availability

Readers may access the data underlying the findings of the study by writing to the corresponding author.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Review Article

Sepsis and Oxidative Stress in the Newborn: From Pathogenesis to Novel Therapeutic Targets

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Sepsis is at present one of the leading causes of morbidity and mortality in the neonatal population. Together with inflammation, oxidative stress is involved in detrimental pathways activated during neonatal sepsis, eventually leading to organ dysfunction and death. The redox cascade during sepsis is mainly initiated by IL-6 and IL-8 stimulation in newborns and includes multiple noxious processes, as direct cell damage induced by reactive oxygen species, activation of gene expression leading to amplification of inflammation and oxidative stress, and impairment of mitochondrial function. Once proinflammatory and prooxidant pathways are established as stimulated by causing pathogens, self-maintaining unfavorable redox cycles ensue, leading to oxidative stress-related cellular damage, independently from the activating pathogens themselves. Despite antioxidant systems are induced during neonatal sepsis, as an adaptive response to an increased oxidative burden, a condition of redox imbalance favoring oxidative pathways occurs, resulting in increased markers of oxidative stress damage. Therefore, antioxidant treatment would exert beneficial effects during neonatal sepsis, potentially interrupting prooxidant pathways and preventing the maintenance of detrimental redox cycles that cannot be directly affected by antibiotic treatment. Among others, antioxidant agents investigated in clinical settings as adjunct treatment for neonatal sepsis include melatonin and pentoxifylline, both showing promising results, while novel antioxidant molecules, as edaravone and endothelin receptor antagonists, are at present under investigation in animal models. Finally, mitochondria-targeted antioxidant treatments could represent an interesting line of research in the treatment of neonatal sepsis.

1. Introduction

Despite general improvement in intensive care of acutely ill newborns, sepsis is still among the leading causes of death in the neonatal population worldwide [1]. On a whole, neonatal sepsis was reported to occur in 1 every 1000 live births [2]; however, incidence as high as 3% to 20% were reported in the population of preterm newborns, due to the presence of multiple coexisting risk factors for nosocomial sepsis [3]. Mortality due to neonatal sepsis is strictly dependent on the causative pathogen and on the gestational age of the patients, with a mortality rate as high as 20% observed in very preterm newborns [2, 3].

According to the guidelines of the International Pediatric Sepsis Consensus Conference, neonatal sepsis is defined as a clinical syndrome characterized by the presence of both infection and systemic inflammatory response syndrome (SIRS) [4, 5]. SIRS includes inadequate core temperature stability, tachycardia or bradycardia, tachypnea or unexplained need for mechanical ventilation, and leukocyte count elevated or depressed for postnatal age [4]. It is at present widely accepted that the infective insult due to the invasion of sterile tissues by pathogens merely represents the initiation of sepsis, while the process leading to the sepsis syndrome is subsequently maintained by a cascade of inflammatory and oxidative mechanisms that, once activated, act independently from the presence of the pathogens themselves [6].

It was demonstrated that, at least in adults, the immune system shows a typical two-phase response during sepsis, characterized by an initial increase of proinflammatory mediators followed by a shift towards anti-inflammatory cytokines, anergy of T-cells, and also apoptosis-induced loss of cells of the adaptive immune system in the most severe cases [7]. Activation of the immune system during sepsis is paralleled by a complex chain of redox events in both adults [8] and newborns [9], which partially differ among the two populations. The redox cascade initiated by immune activation includes generation of consistent amount of reactive oxygen species (ROS) and reactive nitrogen species (RNS), activation of DNA transcription processes, and mitochondrial functional impairment, eventually leading to multiple organ dysfunction and death [8, 9].

2. Redox Status in Neonatal Sepsis

2.1. The Redox Sepsis Cascade. While tumor necrosis factor-alpha (TNF-alpha) plays a pivotal role in the onset of adult sepsis [8], interleukin- (IL-) 6 and IL-8 represent the cytokines mainly involved in the initiation of the sepsis cascade in the newborn [9]. Levels of IL-6 and IL-8 are significantly increased in septic newborns in comparison to healthy controls, in both early-onset (EOS) and late-onset sepsis (LOS) [10, 11] at least for the first 12–24 hours from the onset of sepsis [10], and were proposed as useful markers for the early diagnosis of sepsis in newborns in experimental settings, and, when available, also in clinical practice [11, 12]. However, recent evidences suggest that the cytokines expression profile may consistently differ among septic newborns, according to the timing of sepsis presentation and to the patients' gestational age. In a small cohort of term and late preterm newborns, TNF-alpha increased in both EOS and LOS in comparison to healthy controls, but only patients with LOS showed also increased levels of IL-6 and IL-10 [13]. Moreover, while proinflammatory cytokines as TNF-alpha and IL-6 were upregulated in the acute phase of sepsis, anti-inflammatory cytokines as IL-4 and IL-10 were preferentially overexpressed during the subacute phase [13], providing evidence that the two-phase immune response, typical of adult sepsis, would likely occur also in newborns. The comparison of immune response during sepsis in preterm newborn ≥ 32 or <32 weeks of gestational age showed that mediators of innate immune response, as C-reactive protein (CRP) and SC5b-9, are increased in both groups, but proinflammatory cytokines as interferon-gamma, TNF-alpha, and IL-6 are upregulated only in the subgroup with gestational age ≥ 32 weeks, while both groups showed increased levels of anti-inflammatory cytokines, as IL-4 and IL-10 [14]. The differential profile of cytokines expression during sepsis suggests that partially different pathways could be involved in the initial trigger of the sepsis process at different gestational ages.

It has been proposed that, following the release of proinflammatory cytokines, several oxidative stress-related pathways are activated through different mechanisms, triggering the initiation of a self-maintaining "sepsis redox cycle," finally leading to cell oxidative damage and mitochondria impairment [9]. According to this model, the cytokine-induced activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) constitutes the first step of the process [9]. Several observations are consistent with NF-κB activation during neonatal sepsis [15, 16], and in a mouse model of *group B Streptococcus* neonatal sepsis, the pathway mediated by c-Jun N-terminal kinase was demonstrated to play a pivotal role in the orchestration of inflammation during sepsis, as its inhibition significantly suppressed proinflammatory cytokines production [17]. Similarly, it was recently demonstrated that protein kinase D is essential for NF-κB pathway activation during *group B Streptococcus* sepsis [18]. NF-κB acts as a powerful transcription factor which binds to DNA and activates the transcription of several different genes including inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) [8, 9], leading to increased production of nitric oxide (NO) and superoxide, respectively [9]. Superoxide levels are further increased by the cytokine-induced direct activation of NADPH oxidase [9]. In a cardiomyocyte model of neonatal sepsis, challenge with Gram-negative lipopolysaccharide (LPS) induced NADPH overexpression, leading to COX-2 overexpression through a MAP-kinase/NF-κB-dependent mechanism [19]. Therefore, both direct activity of NADPH oxidase and NADPH-induced COX-2 upregulation can contribute to increase cytoplasmic superoxide [6]. Superoxide is then dismutated to H_2O_2 by cytoplasmic CuZn-superoxide dismutase (SOD) [6]. In a neonatal mouse model of sepsis-induced lung injury, LPS-induced lung cytokines expression, neutrophils influx, and NF-κB translocation were suppressed in NADPH oxidase-deficient animals [20]. Consistent with these observations, also LPS-induced matrix metalloproteinase expression was reduced, as well as alveolar adverse remodeling characterized by reduced number of alveoli and complexity of lung alveolarization [20]. These observations suggest that NADPH oxidase may play a key role in determining the disruption of lung architecture, typical of bronchopulmonary dysplasia (BPD) [21] in septic preterm newborns.

Increased levels of NO were demonstrated in neonatal sepsis [22, 23]. Particularly, preterm newborns < 27 weeks of gestational age presented lower basal levels of NO in comparison to more mature patients, but produced larger amounts of NO during the first 2 days of bacteremia, suggesting that both the basal production of NO and the modulation of NO production may be related to gestational age [23]. In a cohort of term or near term newborn with EOS, circulating NO was significantly higher in comparison to controls [24]. Moreover, iNOS-deficient mice model presented lower degree of inflammation following exposure to *Escherichia coli* [25], and iNOs overexpression was also demonstrated by real-time PCR in neonatal respiratory epithelial cells challenged with *Staphylococcus aureus* and *Staphylococcus epidermidis*, together with proinflammatory cytokines upregulation [26].

NO, along with ROS, directly inhibits electron transport chain in the mitochondria [27, 28], resulting in impaired energy production and accumulation of mitochondrial superoxide [9, 27, 28]. Mitochondrial dysfunction was

demonstrated to be the central core of deleterious proinflammatory and prooxidant routes in adult sepsis [8]. Within the mitochondrion, superoxide can react with NO to produce peroxinitrite, which in turn decomposes to hydroxyl radical and nitrogen dioxide [9]. These highly reactive species further affect mitochondrial functionality [9], and in cellular model of neonatal sepsis, peroxinitrite was shown to suppress mitochondrial function [29], thus favoring the maintenance of a detrimental pathway within the mitochondrion itself. Alternatively, superoxide anion derived by dysfunctional electron transport chain (ETC) can undergo dismutation to H_2O_2 by MnSOD within the mitochondrion, which is then released in the cytosol [9]. Therefore, as a result of NADPH oxidase activity, COX-2 overexpression, and dysfunctional mitochondrial ETC, increasing amounts of H_2O_2 accumulate in the cytosol and activate NF-κB, thus completing a detrimental self-maintaining redox loop [9].

In preterm newborns, oxidative pathway activation during sepsis would interact with a preexisting prooxidant state. Increased ROS production was demonstrated in preterm newborns, resulting from hyperoxic events and mechanical ventilation [30–32], and prematurity is well known to be associated with increased risk of oxidative stress-related diseases, as BPD and retinopathy of prematurity [31]. On the other hand, impaired antioxidant capacities were demonstrated in preterm newborns, both because of inappropriate interruption of placental-fetal transfer of antioxidant molecules and insufficient endogenous production [32]. In fact, fetal levels of antioxidant enzymes (AOEs) as SOD, catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) progressively increase during gestation, paralleling the maturation of surfactant system and preterm animal models fail to induce SOD and GPx in response to oxidative challenges [33, 34], in contrast to term newborns, who were demonstrated to induce AOEs in case of fetal distress [35] or resuscitation with high FiO_2 [36]. The imbalance of the redox status favoring prooxidative pathways in preterm newborns during sepsis could also be implicated in the pathogenesis of BPD and of the long-term neurodevelopmental sequelae observed in this population following a septic event [37]. Despite the exact pathogenetic mechanisms leading to long-term adverse outcomes in septic newborns remain to be elucidated, it has been proposed that both direct increase in oxidative burden and activation of apoptosis would play a major role in tissue impairment [8]. During ongoing development of the central nervous system, a fall in ATP levels and an increase of prooxidant species, resulting from deleterious redox cycles, would critically affect the functionality and permeability of the mitochondria [38]. Particularly, the release of cytochrome c from the mitochondria to the cytosol would activate apoptosis through caspase-mediated signaling pathways [39]. Noticeably, caspases, elements of the proapoptotic Bcl-2 family, and the apoptotic protease activating factor 1 were demonstrated to be overexpressed in neonatal brain tissue in comparison to adult brain tissue [39], indicating preferential activation of proapoptotic pathways in newborns following a noxious stimulus. Oligodendrocyte progenitors and subplate neurons, which are the cell species predominantly involved in the pathogenesis of white matter injury and periventricular leukomalacia in newborns, were proved particularly vulnerable to oxidative stress [40, 41]. Apoptosis of such cell species would also be implicated in the occurrence of visual impairment, as it would detrimentally affect the development of the optic nerve and of the visual cortex [40, 42]. Analogously, oxidative damage and apoptosis have been proposed to be involved in the pathogenesis of long-term respiratory morbidities, as BPD, since increased oxidative burden and exaggerated aspects of apoptosis were demonstrated in newborns with respiratory diseases [43–45]. In lung tissue, activation of apoptosis would result in arrest of the normal process of alveolarization, which is the typical lesion of new BPD [21].

Despite mitochondrial impairment has not been specifically investigated in the newborn, such mechanism could be relevant in the setting of neonatal sepsis. In animal models, neonatal endotoxemia was associated with impairment of carnitine palmitoyltransferase I, the enzyme that controls the entry of fatty acids into the mitochondrion and the rate of fatty acid oxidation, in the developing heart and kidney [29]. The activity of the enzyme measured in mitochondria isolated from the heart, but not from the kidney, of septic newborn rats was significantly impaired, likely because of deleterious effects of ROS on ETC [29]. Moreover, treatment with glutamine, an antioxidant agent that increases Krebs cycle intermediates and supports oxidative phosphorylation, was proved effective in reducing the circulating levels of TNF-alpha and IL-10 in newborn rats exposed to LPS, although it exerted no effect on lipid peroxidation markers and NO production [46]. These results are consistent with the previous observation of restored mitochondrial ultrastructure following exposure to glutamine treatment in a cellular model of neonatal sepsis [47]. The aspect of mitochondrial dysfunction during sepsis could be of major importance in the population of preterm newborns, as they exhibit differential basal mitochondrial functionality in comparison to term newborns [48]. Particularly, the activity of complex III and IV of respiratory chain, pyruvate dehydrogenase and citrate synthase measured on muscle mitochondria obtained from autopsy, was proved to be markedly lower in preterm newborns in comparison to older children, suggesting an age-dependent functionality of mitochondrial respiratory chain [48], which would expose more preterm newborns to an increased risk of energy deficit, and thus organ failure, particularly in case of superimposed mitochondrial impairment during sepsis.

2.2. Markers of Oxidative Stress and Antioxidant Defense. The profile of circulating markers of oxidative stress and of enzymatic and nonenzymatic antioxidant defenses during neonatal sepsis has been less extensively studied in comparison to adult patients (Table 1). In septic newborns, circulating levels of TNF-alpha and malondialdehyde (MDA), a common marker of polyunsaturated fatty acid peroxidation, were shown to be significantly increased in comparison to healthy controls [49, 50] along with antioxidant enzymes xanthine-oxidase, SOD, and GPx, while peroxidase and uric acid levels were suppressed [49]. Moreover, GPx in the subset

Table 1: Clinical studies assessing oxidative stress and/or antioxidant defenses in neonatal sepsis.

Subjects	Evaluated markers	Main findings	Ref.
50 newborns: 30 sepsis/20 controls	Serum XO, CPK, SOD, GPx, PO, MDA, uric acid, albumin	Increased XO, CPK, SOD, GPx, MDA, reduced PO, uric acid, albumin in sepsis versus controls	[49]
50 newborns: 30 sepsis/20 controls	Serum TNF-alpha, SOD, GPx	Increased TNF-alpha, SOD, GPx in sepsis versus controls 5-fold increase of TNF-alpha but no differences in SOD, GPx in septic shock versus nonseptic shock	[50]
128 newborns: 44 sepsis/84 controls	Serum MDA, SOD, GPx, CAT, uric acid, albumin	Increased MDA, SOD, GPx, CAT, reduced uric acid, albumin in sepsis versus controls Increased MDA, SOD, GPx, CAT, reduced uric acid, albumin in sepsis-related death versus sepsis survivors	[51]
120 preterm newborns: 20 proven EOS/20 clinical EOS/80 controls Mean GA: 30 wks	Cord blood IL-6, IL-10-TBARS, protein carbonyls	Increased IL-6, IL-10, TBARS, protein carbonyls in proven and clinical sepsis versus controls Increased TBARS, IL-6 in proven versus clinical sepsis TBARS and IL-6 best biomarkers for the diagnosis of sepsis (AUC 0.88) TBARS is the only marker independently associated with EOS	[54]
120 preterm newborns	Serum IL-6, IL-10, TBARS, protein carbonyls	IL-6 and TBARS showed mild to moderate correlation with sepsis severity score No markers predict sepsis-related mortality	[55]
30 term newborns: 20 sepsis/10 controls	Serum MDA + 4-HDA	2-fold increase of MDA + 4-HDA in septic patients versus controls	[63]
52 newborns with LOS: 27 clinical/25 proven LOS Mean GA: 35 wks	Erythrocyte GPx, TrxR, SOD, CAT, selenium, and glutathione; SePP; plasma lipid peroxidation markers	Increased GPx in clinical sepsis versus controls: Reduced TrxR in proven sepsis versus controls: Increased SOD, CAT, lipid peroxidation, reduced selenium, SePP, glutathione in clinical and proven sepsis versus controls	[64]
70 newborns: 35 LOS/35 controls Mean GA: 36 wks	Serum PON-1, TOS, TAS, OSI	Increased TAS/TOS/OSI; reduced PON-1 in sepsis pretreatment versus posttreatment. Increased TAS/TOS/OSI; reduced PON-1 in sepsis pretreatment versus controls	[65]
65 preterm newborns 31 sepsis/34 controls Mean GA: 34 wks	Erythrocyte SOD, CAT, GPx, GR	Increased CAT at 60 days following sepsis diagnosis in sepsis versus controls	[68]

XO: xanthine-oxidase; CPK: creatinine phosphokinase; SOD: superoxide dismutase, GPx: glutathione peroxidase, PO: peroxidase; MDA: malondialdehyde; CAT: catalase; BAP: biological antioxidant potentials; TBARS: thiobarbituric acid reactive species; 4-HAD: 4-hydroxylalkenals; TrxR: thioredoxin reductase; PON-1: paraoxonase-1; GR: glutathione reductase; SePP: selenoprotein P; TOS: total oxidant state; TAS: total antioxidant state; OSI: oxidative stress index.

of newborns with septic shock was significantly higher than in patients with sepsis but no septic shock [49], suggesting a preferential hyperactivation of antioxidant pathways related to glutathione during septic shock. In 44 newborns with sepsis, MDA, SOD, GPx, and CAT were significantly increased in comparison to controls, while uric acid and albumin levels were significantly reduced [51], and similar changes were also observed in those newborns who died because of sepsis in comparison to survivors [51].

These data are consistent with the activation of prooxidant pathways and ROS overproduction during sepsis, paralleled by an increased activity of antioxidant defense systems, which, however, cannot cope with increased oxidative burden, resulting in detrimental cellular effects, as demonstrated by increased markers of oxidative damage [49]. In accordance with these observations, in a neonatal sepsis model obtained by cecal ligation and perforation (CLP) in piglets, total hydroperoxide (TH) and biological antioxidant potentials (BAP) were both increased 1 hour after the procedure in comparison to sham animals, and BAP remained significantly higher during the 6-hour study period [52]. The increase of TH and BAP was paralleled by a significant increase of TNF-alpha and IL-6 in septic animals in comparison to controls, and a positive correlation was observed at 1-hour post-CLP between TH and BAP, TH and TNF-alpha, and BAPs and IL-6 [52], suggesting mutual interactions between inflammatory pathways and oxidative stress during neonatal sepsis. Consistent with these data, more recently, excessive ROS production was directly demonstrated by DCF fluorescence technique in vital section of renal cortex of newborn rats exposed to LPS [53].

In a cohort of 120 preterm newborns with mean gestational age of 31 weeks, including 20 patients with proven EOS, 20 patients with highly probable EOS, and 80 uninfected controls, oxidative stress and inflammatory markers in cord blood samples were proved to be significantly higher in septic patients versus controls [54]. Particularly, both protein carbonyls, a marker of protein oxidation, and thiobarbituric acid reactive species (TBARS), a marker of lipid peroxidation, were increased along with IL-6 and IL-10 levels in patients with sepsis, both proven or clinically highly probable, in comparison to controls, and in patients with proven sepsis in comparison to controls [54]. Only TBARS and IL-6, but not the other markers, were significantly increased in the group of proven sepsis versus highly probable sepsis [54]. According to ROC curve analysis, TBARS and IL-6 showed the best performance for the diagnosis of EOS among the studied markers with an area under the curve of 0.88. Multivariate logistic analysis comparing TBARS and IL-6 showed that TBARS is a better predictor of EOS and TBARS was the only marker independently associated with EOS [54]. TBARS and IL-6 levels in preterm newborns also showed a mild to moderate correlation with clinical sepsis severity score, although no correlation was demonstrated between these markers and sepsis-related mortality [55]. As some markers of oxidative stress, along with indicators of antioxidant defense, are available as point-of-care test (POCT), the confirmation of a relationship between oxidative stress markers and sepsis severity would be of major relevance in critical care settings. Particularly, available or under development POCTs for oxidative stress and antioxidant status include free oxygen radicals test [56, 57], free oxygen radicals defense test [56, 57], 8-hydroxy-2′-deoxyguanosine [58], 3-nitrotyrosine [59], CuZn SOD [60], BAP, measured as capacity of reduction of ferric to ferrous ions [57], and iridium-reducing assay, particularly sensitive to GSH [61]. Despite at present these POCTs have been studied in the adult population, they would offer several advantages for critically ill newborns, as timely manner stratification of sepsis severity and identification of a patient who would particularly benefit from antioxidant strategies, the need for small blood samples, and the possibility to monitor the response to antioxidant treatment. At present, no relationship has been established between oxidative stress markers and the development of long-term adverse outcomes in septic newborns; however, some biomarkers of inflammation, as S100B, adrenomedullin, and neuron-specific enolase, were proved to be also markers of neonatal brain damage [62]. Moreover, cord blood levels of oxidative stress markers were related to free radical-related diseases, including IVH, in preterm newborns [31], indicating that studies are needed in order to assess whether early markers could predict long-term outcome in septic newborns.

In a small cohort of septic term newborns, pretreatment levels of lipid peroxidation-derived aldehydes, MDA and 4-hydroxylalkenals (4-HDA), were demonstrated to be roughly 2-folds higher than in healthy controls [63]. In 52 newborns with LOS and mean gestational age of 35-36 weeks, plasma lipid peroxidation markers and protein carbonyls were proved to increase significantly in patients with proven or clinical sepsis in comparison to uninfected controls [64]. The study of erythrocyte selenoenzymes showed increased GPx levels in patients with clinical sepsis and reduced thioredoxin reductase levels in patients with proven sepsis in comparison to controls [64], demonstrating differential regulation of antioxidant selenoenzymes during sepsis. SOD and CAT were increased in septic patients, demonstrating an adaptive antioxidant response to oxidative stress during sepsis, while erythrocyte selenium, erythrocyte glutathione, and selenoprotein P, the main plasma selenoprotein, were markedly decreased in patients with proven or clinical sepsis in comparison to controls, suggesting consumption of selenium-containing antioxidant molecules [64].

According to the available evidence, in neonatal sepsis, both oxidative stress-related pathways and antioxidant defenses appear induced; however, redox unbalance favoring oxidative stress likely occurs, as markers of oxidative damage are increased in comparison to controls [49, 50]. Concordant with this observation, in 70 newborns with mean gestational age of 36 weeks, total oxidant state (TOS) and total antioxidant state (TAS) were both increased in septic patients in the pretreatment period versus controls and oxidative stress index (OSI), and the percentage ratio of TOS/TAS, was also increased [65], confirming the prevalence of oxidative stress pathways on antioxidant defense. TOS and TAS were also studied to monitor treatment and significantly decreased

after treatment in septic patients in comparison to pretreatment levels. Moreover, paraoxonase-1 (PON-1), an enzyme located in HDL inhibiting lipoprotein oxidation in LDL [66], which is reduced in adult sepsis [67], appeared significantly lower in septic newborns before treatment in comparison to controls and also to posttreatment levels [65]. In septic patients after treatment, higher TAS levels were observed in comparison to controls, while TOS and PON-1 did not significantly differ, suggesting that compensatory antioxidant defense might continue beyond the initial oxidative burst. In contrast with these findings, in a cohort of preterm newborns with mean gestational age of 34 weeks, no differences in erythrocyte GPx, GR, and CAT were detected between septic patients and controls during the clinical course of sepsis, although in septic patients at 60 days, CAT activity was significantly increased in comparison to controls and GPx activity depressed in comparison to day 0 [68].

Chorioamnionitis is a well-known risk factor for fetal and neonatal infection, especially in preterm newborns, as about 5–17% of preterm newborns whose mother has chorioamnionitis develop EOS [69, 70]. In preterm newborns with gestational age < 30 weeks, oxidative stress markers, as isoprostanes, nonprotein-bound iron, and advanced oxidative protein products were proved to be significantly increased in cord blood of newborns of mothers with histological chorioamnionitis in comparison to the control group [71]. Moreover, a significant positive correlation was found in multivariate analysis adjusted for the main neonatal and perinatal variables between histological chorioamnionitis and cord levels of oxidative stress markers, indicating increased fetal oxidative burden during intra-amniotic infection [71]. Moreover, increased levels of oxidative markers as prolidase, matrix metalloproteinases, TOS, and OSI were demonstrated in vaginal washing fluid of healthy pregnant women with preterm premature rupture of membranes (PPROM) in comparison to controls with intact membranes, while antioxidant parameters, as PON-1 and total antioxidant capacity (TAC), were significantly lower [72]. Moreover, prolidase, matrix metalloproteinases, and oxidative-antioxidant status parameters significantly differed in women with chorioamnionitis in comparison to those without chorioamnionitis in the PPROM group and levels of prolidase, matrix metalloproteinase-13, TOS, TAC, and PON-1 were proved to predict chorioamnionitis in the PPROM group [72]. These results are partially discordant with the observation of measurable amount of oxidative stress markers in amniotic fluid in 183 pregnant women with PPROM but the absence of influence of intra-amniotic infection or histological chorioamnionitis on the levels of oxidative stress and antioxidant biomarkers [73]. Concordantly, intra-amniotic infection, histological chorioamnionitis, and funisitis did not significantly affect cord blood TAC, ferric reducing antioxidant power, TBARS, advanced glycation end products, and markers of oxidative stress in the offspring of 165 pregnancies complicated by PPROM [74]. On a whole, despite increased fetal and oxidative burden could occur as a result of PPROM and chorioamnionitis, although mixed results were reported, at present, no conclusions can be derived regarding its role in neonatal sepsis, as no data on oxidative balance are available for the subset of newborns who develop EOS as a consequence of maternal chorioamnionitis.

2.3. Intestinal Microbiota and Oxidative Stress. The gastrointestinal system was recently demonstrated to take part in the oxidative burst during sepsis in preterm newborns, providing evidence that host-microbiota interactions could be of major importance under septic conditions [75]. Fecal samples of 5 pairs of twins with mean gestational age of 30 weeks, each pair including one septic and one control twin, were used for microbiota analysis and genome-wide expression analysis on exfoliated intestinal cells. Induction of several genes involved in proinflammatory and prooxidant pathways was demonstrated in intestinal cells of septic newborns in comparison to controls, and such genome expression changes were paralleled by microbiota shift towards predominance of *Enterobacteria* with reduction of *Bacteroides* and *Bifidobacteria*, likely resulting from oxidative stress and low-grade inflammation in the gut mucosa [75]. A significant inverse correlation was observed between *Bacteroides* and *Bifidobacteria* and 8 genes involved in oxidative stress, and also further genes involved in TNF-alpha and IL-1beta signaling pathways [75]. These results were in agreement with a previous study of blood genome-wide expression profile in very low birthweight (VLBW) infants, demonstrating overexpression of genes involved in innate immunity and inflammation in septic patients in comparison to controls [76]. These aspects could be of major importance in the population of preterm newborns, who exhibited basal overexpression of genes related to inflammation in the gut in comparison to term newborns, as demonstrated by whole genome sequencing of stool-derived mRNA [77], and overexpressed in septic condition pathways related to IL-1 receptor kinase 2, fibroblast growth factor receptors, gap junctions, and cell division regulators [78]. Moreover, in a preterm pig model of necrotizing enterocolitis (NEC), the study of intestinal proteomics demonstrated that antibiotic treatment induced several beneficial mucosal pathways, including antioxidant ones, as CAT activity was significantly increased in comparison to untreated animals, suggesting that antibiotic treatment during NEC is associated to a more favorable redox profile, shifting towards antioxidant prevalence [79].

Intestinal microbiota has recently become a central component of the sepsis process in preterm newborns as the disruption of physiological intestinal colonization induced by aggressive antibiotic treatment was proved to favor the development of pathogen species and to be associated with adverse outcomes [80, 81]. Early empirical antibiotics administered for more than 5 days, without evidence of positive blood culture, was positively associated to increased risk of NEC and death in a large cohort of ELBW infants [82] and to increased incidence of LOS and of the combined outcome LOS-NEC-death in 365 VLBW infants [83]. It was hypothesized that high levels of circulating proinflammatory and prooxidant mediators observed in septic newborns, through an inflammatory organ cross-talk, may affect gut mucosa gene expression profile, leading to local environment inflammation and oxidative stress that, in

turn, would affect microbial colonization, favoring pathogen species [75].

As antibiotics are one of the milestones of medical treatment of NEC but inappropriate antibiotic courses are related to adverse outcomes, the optimal antibiotic regimen for patients with NEC is of crucial importance in clinical settings [84, 85]. Two recent surveys reported high variability among different centers and within single centers in antibiotic treatments for NEC in terms of type and number of antibiotics and duration of treatment [84, 85]. Despite the most frequently reported regimen was the association of amoxicillin or ampicillin, gentamycin, and metronidazole [84, 85] basing on old data [86], the criteria to broaden antibiotic spectrum were variable among practitioners and for surgical patients, the duration of postsurgery antibiotic course was not standardized [85]. Two meta-analyses found insufficient evidence to make specific recommendations on the most appropriate type and duration of antibiotic treatment [87, 88]; therefore, specifically designed studies should be performed to address the optimal antibiotic regimen for patients with NEC in terms of harm/benefit ratio.

Finally, different antioxidant treatments were suggested to be effective in the prevention of NEC development in preterm newborns. Particularly, oral supplementation with lactoferrin was proved to reduce NEC occurrence in a meta-analysis [89, 90], although this evidence had low-to-moderate quality [91] and human recombinant lactoferrin was administered only in one study, while bovine lactoferrin was used in all the others [91]. The beneficial effect of lactoferrin in preserving the gut mucosa integrity is likely related to the position of lactoferrin itself on the mucosal surface, where it contrasts microbial invasion and translocation across the intestinal wall [91]. On the other hand, pentoxifylline administration showed mixed result, and in meta-analysis, did not affect NEC occurrence [92]. However, because of the low quality of evidence, specific trials investigating pentoxifylline treatment for NEC prevention and treatment are advocated [92]. N-Acetylcysteine administration in a rat model of NEC reduced gut oxidative stress damage measured as MDA, gut abnormalities, and intestinal levels of TNF-alpha, while was proved to increase local activity of antioxidant enzymes [93]. Finally, also melatonin was hypothesized to confer protection against NEC development, due to its pleiotropic and multiorgan antioxidant activities [94]; however, its potential usefulness in NEC prevention remains at present to be assessed by clinical trials.

3. Antioxidant Strategies in Neonatal Sepsis

Basing on evidence of increased oxidative burden in neonatal sepsis, therapeutic strategies targeting proinflammatory and prooxidant pathways would be expected to be beneficial; however, despite promising results in cellular and animal models, evidence from clinical trials is still limited. In the neonatal populations, antioxidant treatments investigated during sepsis include both direct antioxidant administration and pharmacologic inhibition of prooxidant pathways.

Melatonin demonstrated pleiotropic antiapoptotic, antioxidant, and anti-inflammatory effects in vitro and in vivo, as direct scavenging activity against ROS and other oxidizing agents and stimulation of antioxidant enzymes, as CAT, SOD, GPx, GR, and gamma-glutamylcysteine synthase, the rate-limiting enzyme in glutathione synthesis [95]. Interestingly, melatonin accumulates within the mitochondria [95, 96]; therefore, it would possibly target the local excessive ROS production, which is typical of dysfunctional mitochondria during sepsis [8, 27]. In preterm newborns, melatonin was demonstrated effective in reducing oxidative stress markers and inflammatory mediators in RDS and perinatal asphyxia [97, 98]. In a small cohort of septic term newborns, oral melatonin treatment within the first 12 hours from diagnosis (2 doses, 10 mg/kg each, administered at 1-hour interval) significantly reduced lipid peroxidation markers, MDA+4-HDA, at 1 and 4 hours after treatment [63] (Table 2). Melatonin-treated infants also showed a significant reduction in white blood cell count, absolute neutrophil count, and CRP 24 hours after treatment, while at the same time untreated septic infants presented stable white blood cell count and neutrophil count and increased CRP levels. Moreover, while 3 of 10 septic untreated infants died, no cases of death were observed in the melatonin-treated group [63]. These encouraging results are consistent with a favorable suppression of prooxidant and proinflammatory pathways induced by melatonin treatment in neonatal sepsis, occurring as early as 1 hour after oral administration [63]. Concordantly, in a recent nonrandomized trial including 40 septic newborns treated with antibiotics and melatonin (20 mg/kg, single dose) or antibiotics alone, melatonin treatment was associated with a significantly stronger reduction of CRP levels and improvement of clinical parameters in comparison to antibiotic treatment alone [99] and it was also shown to improve clinical sepsis score in comparison to antibiotic treatment alone in a cohort of 50 septic newborns [100]. These data are also concordant with the observation of beneficial effect of melatonin in newborns in the postsurgery period, showing reduced nitrite-nitrate and proinflammatory cytokines levels following melatonin administration in comparison to untreated surgical newborns [101]. Interestingly, higher melatonin levels were demonstrated in septic newborns with LOS in comparison to uninfected controls [102], suggesting that melatonin endogenous production might be upregulated during sepsis, taking part in antioxidant defense. Basing on these favorable preliminary data, randomized control trials are warranted to assess efficacy and safety of melatonin as an adjunct treatment in neonatal sepsis [103].

Promising results in antioxidant treatment for neonatal sepsis were obtained with the administration of pentoxifylline, which exerts several antioxidant and anti-inflammatory activities, as reduced glutathione level restoration, mitochondrial viability maintenance, inhibition of TNF-alpha production, preservation of proper endothelial function and of proper coagulation activity, and prevention of gastrointestinal vasoconstriction [104]. In a randomized controlled trial including 120 newborns with LOS and mean gestational age of 30 weeks, pentoxifylline administration (5 mg/kg/h i.v. for 6 hours for 6 days) was associated with reduced TNF-alpha and CRP levels, reduced need for vasopressor, shorter duration of respiratory support and

Table 2: Main evidence from clinical studies on melatonin and pentoxifylline treatment in neonatal sepsis.

Enrolled population	Interventional procedure	Outcomes	Ref.
Melatonin			
30 newborns: 10 sepsis/10 sepsis and melatonin treatment/10 controls	Melatonin, 20 mg/kg orally within 12 hours of sepsis diagnosis (2 doses, 10 mg/kg each, separated by 1-hour interval)	Reduced MDA + 4-HDA at 1 and 4 hours after treatment in septic treated versus septic untreated infants Reduced WBC count, ANC, CRP 24 hours after treatment in septic treated versus septic untreated infants	[63]
40 newborns: 20 sepsis/20 sepsis and melatonin treatment	Melatonin, 20 mg/kg orally, single dose	Reduced CRP and better clinical improvement at 24 and 72 hours after treatment in treated versus untreated infants	[99]
50 newborns: 25 sepsis/25 sepsis and melatonin treatment	Melatonin, 20 mg/kg orally, single dose	Reduced sepsis score at 24 and 48 hours after treatment in treated versus untreated infants	[100]
Pentoxifylline			
120 newborns: 60 LOS/60 LOS and pentoxifylline treatment	Pentoxifylline, 5 mg/kg/h IV for 6 hours for 6 days	Reduced TNF-alpha, vasopressor need, duration of respiratory support, duration of antibiotics, hospital stay, incidence of DIC, and thrombocytopenia in treated versus untreated infants No differences in mortality	[105]
Meta-analysis of 6 randomized or quasi-randomized trials; 416 newborns	Pentoxifylline, continuous IV infusion, different dosing regimens	Reduced all-cause mortality, reduced hospital stay in septic treated versus untreated septic infants Reduced mortality in the subgroup of preterm newborns, proven sepsis, and Gram-negative sepsis in septic treated versus untreated septic infants	[106]

MAD: malondialdehyde; 4-HDA: 4-hydroxylalkenals; WBC: white blood cell; ANC: absolute neutrophil count; CRP: C-reactive protein; DIC: disseminated intravascular coagulopathy; LOS: late-onset sepsis.

antibiotic treatment, shorter hospital stay, lower incidence of disseminated intravascular coagulopathy, metabolic acidosis, and thrombocytopenia [105] (Table 2). However, no differences were observed in mortality and short-term morbidity between pentoxifylline-treated and untreated septic newborns [105, 106]. In a meta-analysis including 6 small studies, pentoxifylline administration in septic newborns was proved effective in reducing all-cause mortality and the length of hospital stay, and subgroup analysis demonstrated significantly reduced mortality in preterm infants, in infants with proven sepsis, and in infants with Gram-negative sepsis [92], leading to the conclusion that pentoxifylline may represent a beneficial adjunct treatment in neonatal sepsis, although larger trials are needed in order to define pentoxifylline efficacy and the safety profile. Interestingly, in vitro pentoxifylline was recently proved to exert more powerful anti-inflammatory effects in newborns than in adults [107]. In cord blood and adult blood stimulated with LPS, pentoxifylline treatment suppressed TLR-mediated cytokines levels, as TNF-alpha and IL-1beta, in a dose-dependent manner and this effect was more pronounced in cord blood in comparison to adult blood [107], suggesting that pentoxifylline adjunct treatment could be particularly beneficial in the neonatal population.

Lactoferrin, a normal component of human milk, is an anti-infective and antioxidant agent, acting through iron sequestration and direct detrimental effect on pathogen cell membranes [108]. In a randomized controlled trial including 472 VLBW infants who were randomized to lactoferrin alone or lactoferrin and probiotics or placebo, the supplementation with lactoferrin alone or in combination with probiotics significantly reduced the incidence of LOS, both fungal and bacterial, in comparison to placebo [109]. Moreover, a recent meta-analysis including 6 randomized controlled trials showed that lactoferrin supplementation to enteral feeds in preterm newborns, alone or in combination with probiotics, reduced the incidence of LOS and NEC stage II or III, although overall mortality was not affected [89]. However, at present, no evidence are available on the possible beneficial effect of lactoferrin administration during neonatal sepsis; therefore, further studies are needed to assess whether lactoferrin could be beneficial not only as a preventive measure but also as an adjunct treatment for neonatal sepsis and also to establish the optimal dosing regimen. Interestingly, in 15 preterm newborns, serum lactoferrin levels were significantly lower in patients with proven sepsis in comparison to those with clinical sepsis and were positively correlated with white blood cell count or absolute neutrophil count, suggesting that the lowest lactoferrin observed in more immature infants is likely related to suboptimal white cell activity and that lactoferrin supplementation could be particularly effective in this population [110].

Vitamin E, which acts primarily as circulating direct antioxidant, has been extensively investigated for the prevention of prematurity-related mortality and morbidity and was proved effective in reducing the incidence of intracranial bleeding and retinopathy of prematurity in the subset of VLBW infants in a meta-analysis including 26 randomized

Table 3: Animal studies of novel antioxidant treatments in neonatal sepsis.

Model	Interventional procedures	Outcomes	Ref.
Edaravone			
Piglets	CLP alone or CLP and IV continuous edaravone infusion	Reduced TH at 1 hour after CLP, reduced nitrite-nitrate at 3 and 6 hours, reduced HMGB-1, delayed TNF-alpha surge, increased mean arterial pressure, reduced heart rate, longer survival time in treated versus untreated animals	[117]
Piglets	CLP alone or CLP and IV continuous edaravone infusion	Reduced pulmonary hypertension in treated versus untreated animals Mean pulmonary artery pressure/mean systemic arterial pressure ratio positively related to TNF-alpha levels	[118]
Endothelin-1 receptor antagonist			
Piglets	CLP alone or CLP and IV continuous ETR-P1/fl infusion or controls	Reduced nitrite-nitrate, TNF-alpha, HMBG-1, reduced pulmonary hypertension in CLP-treated animals versus CLP alone	[123]
Piglets	CLP alone or CLP and IV continuous ETR-P1/fl infusion or controls	Reduced TH, OSI, IL-6 at 3 and 6 hours post-LP	[124]

CLP: cecal ligation perforation; TH: total hydroperoxide; OSI: oxidative stress index; HMGB-1: high mobility group box 1.

controlled trials [111]. However, vitamin E supplementation was associated with increased risk of sepsis both in the case of intravenous and nonintravenous administration and serum tocopherol levels higher than the cut-off of 3.5 mg/dL were positively associated with increased risk of sepsis [111], leading to the conclusion that routine vitamin E supplementation in preterm newborns cannot be recommended. In a recent randomized open-label study including 65 preterm newborns with mean gestational age of 34 weeks, vitamin E supplementation reduced GPx activity at 30 days in septic newborns in comparison to untreated septic newborn although it also mitigated the reduction of GPx observed in septic patients 60 days after sepsis onset [68]. Moreover, vitamin E supplementation suppressed GR activity in treated septic patients, while increased GPx activity in controls in comparison to untreated controls [68]. The combination of direct scavenging effect of vitamin E with increased GPx activity would result in enhanced H_2O_2 removal with a reduction in efficiency of circulating pathogen discharge and therefore would explain the increased risk of sepsis observed in vitamin E-supplemented newborns [68, 111].

Other antioxidant measures with potential efficacy in neonatal sepsis that have been investigated in clinical settings include selenium [112] and zinc supplementation [113], and treatment with ibuprofen [114].

Edaravone (3-methyl-1-phenyl-pyrazolin-5-one), a free radical scavenger introduced in the latest years in experimental settings, exerts multiple antioxidant effects, as hydroxyl radical scavenging, suppression of hydroxyl-dependent lipid peroxidation, and electron donation to ROS [115], leading to beneficial effects in animal models of neonatal hypoxic-ischemic encephalopathy [116]. In a piglet model of neonatal sepsis, edaravone was demonstrated to reduce TH levels 1 hour after CLP and nitrite-nitrate levels at 3 and 6 hours in comparison to septic untreated animals, indicating favorable antioxidant effects [117] (Table 3). These changes were paralleled by clinical improvement of septic animals, as demonstrated by higher cardiac output and mean arterial pressure, lower heart rate, and longer survival time in treated versus untreated animals, suggesting possible beneficial effects of edaravone on sepsis clinical course in the newborns [117]. Furthermore, edaravone delayed TNF-alpha surge in treated animals and also prevented the increase of high mobility group box 1 (HMGB-1), a nuclear transcription factor involved in the systemic inflammatory response [117]. In the same animal models, edaravone was also proved effective in reducing sepsis-related pulmonary hypertension and the ratio between mean pulmonary and systemic arterial pressure was positively related to TNF-alpha levels, suggesting that edaravone may exert suppressive action on TNF-alpha release [118]. In a small cohort of pediatric patients with cerebral infarction, edaravone was recently associated with improved neurological outcome without significant adverse effects [119].

The endothelin system is well known to be involved in sepsis, as endothelin-1 (ET-1) induces activation of NF-κB-mediated proinflammatory pathways and expression of adhesion molecules [120] and ET-1 levels appear increased in septic newborns, particularly in case of pulmonary hypertension [121, 122]. The infusion of endothelin receptor antagonist ETR-P1/fl was proved effective in reducing serum nitrite and nitrate, TNF-alpha, and HMBG-1 in a piglet model of neonatal sepsis and also in reducing pulmonary hypertension and increasing mean arterial pressure and survival time [123] (Table 3). In accordance with this study, in the same animal model, treatment with ETR-P1/fl reduced TH, OSI (calculated as total hydroperoxide/biological antioxidant potentials), and IL-6 at 3 and 6 hours after CLP, indicating attenuation of prooxidant and proinflammatory insult [124]. At present, endothelin receptor antagonists have never been investigated in clinical settings in the newborn.

As dysfunctional mitochondria are the key factor of organ impairment during sepsis [8, 28], mitochondrial-targeted antioxidant treatment has been studied in preclinical models of adult sepsis [125]. In order to achieve antioxidant protection of mitochondria, potential useful strategies include the administration of ROS scavengers, which specifically target the mitochondria and act where needed within the mitochondria, or the induction of endogenous mitochondrial antioxidant system [125]. Particularly, the main studied agents are obtained by conjugation of antioxidant molecules

to lipophilic cations that accumulate in the mitochondria, driven by mitochondrial membrane potentials, as MitoQ, containing ubiquinone antioxidant moiety, or by the conjugation of fragment of antibiotic with the stable nitroxide radical TEMPOL, that is able to accept electrons from unstable ROS, to dismute superoxide anion, and to exert catalase-like activity [125]. Other strategies under investigation include the administration of small synthetic peptides (SS peptides) with scavenger activity, which selectively concentrate in the mitochondria, and potentiation of endogenous mitochondrial antioxidant defenses by the administration of N-acetyl-l-cysteine, which accumulates in the mitochondria and increases local glutathione availability or by genetic approaches, as adenoviral transfection with MnSOD [125]. At present, none of these strategies has been studied in models of neonatal sepsis; therefore, mitochondrial-targeted antioxidant treatment could represent a potential future line of research in the field of neonatal sepsis.

4. Conclusions

Oxidative stress, along with proinflammatory pathways, was demonstrated to play a major role in neonatal sepsis both in vitro and in vivo. However, few evidences are available at present on the clinical usefulness of adjunct antioxidant treatment in neonatal sepsis.

Some relevant limitations of the studies investigating oxidative stress and antioxidant treatments in neonatal sepsis may partially limit the quality of the available evidence. Most of the studies assessing antioxidant enzyme activity in neonatal sepsis were based on serum measurements that could be influenced by hemolysis processes and in fact provided partially different evidences in comparison to observations based on erythrocyte level measurements [68]. Moreover, data obtained from animal studies on oxidative stress markers or antioxidant treatments in neonatal sepsis may not strictly reflect the clinical conditions of septic newborns, as in experimental settings, the animal models are challenged with high levels of LPS or bacteria, which is often not the case in clinical practice [52, 53, 117, 118, 123, 124]. As regard to clinical observations, not all of the studies reported rigorous inclusion criteria, defining whether both EOS and LOS or only one of the two categories is included; therefore, conclusions derived from these studies could be misleading as redox pathophysiology of EOS and LOS could be partially different. Moreover, while some studies included only culture-proven sepsis, others included both proven and clinical sepsis, and others did not specify this aspect. However, due to the fact that several nonsepsis-related perinatal variables could affect oxidative stress in newborns, only data coming from proven sepsis patients should be considered fully reliable, while those coming from clinical sepsis patients could merely reflect the effect of different conditions activating oxidative stress pathways. Finally, clinical studies investigating the usefulness of antioxidant treatments in neonatal sepsis often lack strict randomization, leading to evidences that, although promising, need to be confirmed by properly designed studies.

Despite several limitations, available evidence suggests that oxidative stress processes are activated during neonatal sepsis, posing the basis for the potential clinical usefulness of antioxidants as an adjunct strategy for the treatment of septic newborns. Antioxidants, in fact, would counterbalance the detrimental prooxidant cycle in newborn sepsis, which, once initiated, proceeds independently from the pathogens themselves and thus is not affected by antibiotic treatment alone. Further studies are needed to identify useful agents and to standardize antioxidant treatment in neonatal sepsis.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

The Free Radical Diseases of Prematurity: From Cellular Mechanisms to Bedside

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During the perinatal period, free radicals (FRs) are involved in several physiological roles such as the cellular responses to noxia, the defense against infectious agents, the regulation of cellular signaling function, and the induction of a mitogenic response. However, the overproduction of FRs and the insufficiency of an antioxidant mechanism result in oxidative stress (OS) which represents a deleterious process and an important mediator of damage to the placenta and the developing fetus. After birth, OS can be magnified by other predisposing conditions such as hypoxia, hyperoxia, ischemia, hypoxia ischemia-reperfusion, inflammation, and high levels of nonprotein-bound iron. Newborns are particularly susceptible to OS and oxidative damage due to the increased generation of FRs and the lack of adequate antioxidant protection. This impairment of the oxidative balance has been thought to be the common factor of the so-called "free radical related diseases of prematurity," including retinopathy of prematurity, bronchopulmonary dysplasia, intraventricular hemorrhage, periventricular leukomalacia, necrotizing enterocolitis, kidney damage, and oxidative hemolysis. In this review, we provide an update focused on the factors influencing these diseases refining the knowledge about the role of OS in their pathogenesis and the current evidences of such relationship. Mechanisms governing FR formation and subsequent OS may represent targets for counteracting tissue damage.

1. Introduction

Each molecule is characterized by a particular concentration of electrons that establish its own redox state. When specific conditions occur, the redox state can be altered to lower or higher levels thus forming free radicals (FRs) [1]. FRs are highly reactive substances that are capable to start self-amplified chain reactions causing cellular dysfunction and damage. Many antioxidant enzymes exist to counteract this propagation, and when the production of FRs exceeds the capacity of scavenger defenses, an oxidative stress (OS) occurs [2].

In the perinatal period, a properly controlled oxidative species production has been proven to be a necessary factor [3]. After fertilization, the beneficial effects of FRs occur at low/moderate concentrations and involve physiological roles in sperm capacitation, acrosome reaction, sperm-egg interaction, and gamete fusion [4, 5]. Until the beginning of the second trimester, fetal development takes place in a low-oxygen environment presumably to protect the embryo, which is highly sensitive to reactive oxygen species (ROS) [6]. Subsequently, due to the placental maturation, a threefold rise in the oxygen concentration causes an exponential increase of ROS [7]. In this phase, ROS regulate gene transcription and downstream activities such as trophoblast proliferation, invasion, and angiogenesis. OS-induced apoptosis influences placental vascular modifications [8], induces autophagy, and together ensures the normal cellular turnover until the late gestation [7]. The placenta adapts to the increase in ROS generation by modulating hypoxia-inducible factor 1α (HIF-1α) and increasing cellular antioxidant levels [9]. Under normal conditions, such adaptation mechanisms must occur to ensure a proper fetal development [10]. However, in a scenario where OS is abnormally increased, chelation mechanisms may be insufficient and damage affecting both the fetus and the

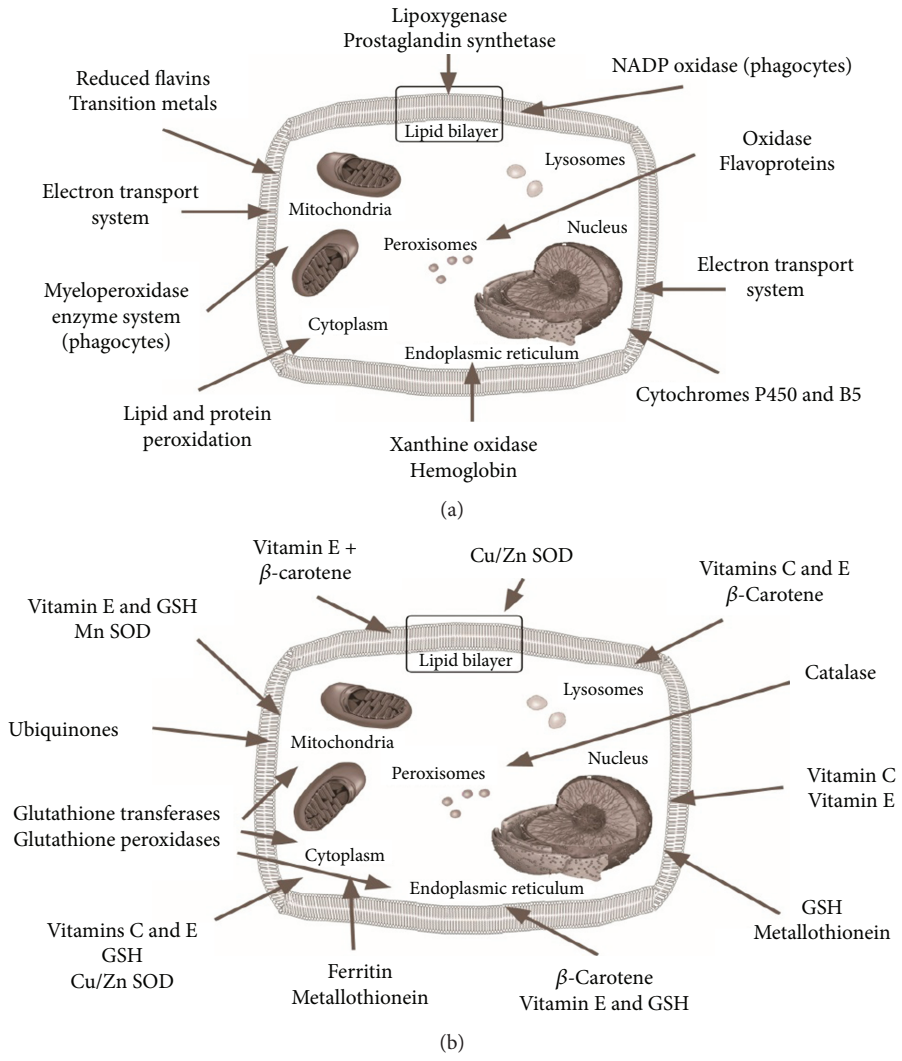

Figure 1: Cellular sources of free radicals (a); antioxidant protection within the cell (b).

mother can be observed. Many studies have shown a relationship between increased OS levels and several pregnancy diseases, including defective embryogenesis, spontaneous abortion, preeclampsia, intrauterine growth restriction, gestational diabetes mellitus, premature rupture of membranes, and minor congenital abnormalities [3, 9, 10–12].

The intrauterine environment and redox state can also play a role for future diseases in the child such as obesity and diabetes mellitus and hypertension in adulthood [13–15]. Finally yet importantly, an increased oxidative level in the perinatal life may trigger a deleterious state of OS in the newborn, especially in preterm infants, which is capable to activate underlying mechanisms that lead to the onset of the so-called "oxidative stress-related diseases in newborn" [16–18]. The present review intends to make a journey into the free radical chemical biology and to update the current knowledge about the role of OS in the pathogenesis of such gestational, fetal, and neonatal diseases.

1.1. Free Radical Chemical Biology. Free radical reactions are a normal occurrence in living organisms, and ROS are deeply involved in signaling molecules to regulate a wide variety of physiological events.

There are different FR species: oxygen-centred radicals (ROS), nitrogen-centred radicals (RNS), carbon-centred radicals, and sulphur-centred radicals [19]. Intracellular generation of ROS can occur as a byproduct with mitochondria, peroxisomes, cytochrome P450, and other cellular elements (Figure 1(a)). ROS generation by mitochondria is a highly variable and depends both from metabolic conditions and the intramitochondrial balance between oxidative and antioxidative factors.

Under physiologic conditions, approximately 98% of O_2 undergoes a complete reduction to form H_2O_2. Approximately, 2% of electrons will leak causing a partial reduction of O_2-producing ROS [20]. The monovalent reduction of O_2 produces superoxide anion (O^-), and monovalent reduction of O^- generates hydrogen peroxide (H_2O_2). A third monovalent reduction generates the highly reactive hydroxyl radical ($OH^\bullet$) [21, 22].

Any damage to the energy-producing machinery of the mitochondria will result in superoxide accumulation, and

any process that results in depletion of antioxidant defense will result in the default conversion of superoxide to even more oxygen reactive species.

Other important FR cellular sources are the following: the activity of monoamine oxidase, which deaminates biogenic amines and produces hydrogen peroxide (H_2O_2); the purine catabolism by xanthine oxidase (XO), producing superoxide anion radical (O_2^-); the Haber-Weiss and Fenton reactions by nonprotein-bound iron (NPBI) producing the hydroxyl radical (OH); peroxynitrite ($ONOO^-$) generator enzymes, such as xanthine oxidoreductase, nicotinamide adenine dinucleotide phosphate oxidase (Nox), nitric oxide synthase (NOS), and heme oxygenase (HO) [23]; and the inflammatory response where the production of H_2O_2 and $O_2^{\bullet-}$ increases in white blood cells and Kupffer cells due to NADPH-dependent oxidase system, coupled to the action of superoxide dismutase (SOD).

Aerobic organisms have developed well-integrated antioxidant defenses to enable them to handle and scavenge FRs [24] (Figure 1(b)). These defenses include antioxidant enzymes and low molecular weight antioxidant compounds like vitamins A, E, and C, beta-carotene, lipoic acid, and glutathione. The antioxidant enzymes, like SOD, catalase, and GPX, have the capacity to scavenge the levels of ROS produced ever in physiological conditions [25]. Under ischemic conditions, these antioxidant enzymes fail to protect tissue from oxidative damage because of the overproduction of oxygen radicals and consumptions of antioxidant defense [25].

Once generated, the condition of OS may perpetuate the damage to all components of the cell, including proteins, lipids, and DNA. F2-isoprostanes (F2-IsoPs) are prostaglandin F2-like compounds derived by the FR peroxidation of arachidonic acid. They are recognized as reliable marker of lipid peroxidation. Docosahexaenoic acid (DHA), a major component in neuronal membranes, oxidizes both in vitro and in vivo to form F2-IsoP-like compounds termed F4-neuroprostanes (F4-NPs) [16, 26–29]. An oxygen insertion step diverts intermediates from the IsoP pathway to form isofurans (IsoFs) that contain a substituted tetrahydrofuran ring. Because of this differential method of formation, it has been focused that oxygen concentration can affect lipid peroxidation profile. Like the IsoPs, the IsoFs are chemically and metabolically stable so are well suited to act as *in vivo* biomarkers of oxidative damage. The NPs are the only quantitative *in vivo* biomarker of oxidative damage that is selective for neurons. An alternative pathway of oxidation of DHA brings to the formation of an IsoF-like compound termed neurofurans (NFs). Quantitative assessment of NFs in vivo reveals modulated formation under conditions of elevated and diminished OS [28]. Proteins are also vulnerable to FR attacks. Similar to lipid peroxides, altered protein molecules, such as protein peroxides and protein-bound reducing moieties, can act as traps for the chemical energy released by FR. Then they can initiate further radical chain reactions, thus enhancing the damage. Advanced oxidative protein products (AOPP) are reliable markers of the degree of protein damage in OS [30, 31].

In term of increased NPBI concentration, the link between increased FR release and OS during fetal/neonatal asphyxia has emerged with reports of increased plasma F2-isoprostanes and advanced oxidative protein products (AOPP), as indices of lipid and protein oxidation, in cord blood [27, 32].

2. Oxidative Stress in the Prenatal Period

Embryos and fetuses have a relative immaturity of the antioxidant system that facilitates the exposure to the damaging effects of an OS condition [12]. ROS highly affect embryo and fetus development, thus causing different diseases with a common pathophysiology based on antioxidant impairment and FR overproduction.

Some conditions of pregnancy are specific triggers for an overload of FRs, thus setting an adverse intrauterine environment with subsequent fetal development impairment [33, 34]. Morphological and immunohistochemical analyses show an increased oxidative damage in placental tissues obtained from early spontaneous abortion compared with normal controls [35]. On the other hand, impaired extravillous trophoblast invasion and insufficient uterine artery remodeling, associated with the onset of preeclampsia (PE), lead to highly resistant spiral arteries and to ischemia/reperfusion of placenta [36, 37]. Oxidase activity was detected and confined to the microvillus membrane of syncytiotrophoblast and might be abnormally regulated in PE pregnancies [33]. A deregulation of phospholipases A2 could potentially be implicated in freeing F2-isoP, which could participate in local hypertension observed in the PE placenta through the thromboxane pathway. Indeed, free F2-isoP was found to be significantly higher in preeclamptic woman than normotensive controls [38]. PE is not only an endothelial disease but also a consequence of a wider range of systemic inflammatory network responses [39]. Activated macrophages, neutrophils, and Th1 cells can infiltrate into renal and other tissues in women with PE [39]. Systemic inflammation and circulating antiangiogenic factors may ultimately end in multiorgan dysfunction if not controlled in a timely manner [40].

Early-onset PE is almost invariably associated with IUGR [34]. Despite having distinctive clinical manifestations, there is an accumulating evidence that the two pathologies have a common cause: an abnormal placental implantation [41]. This deficiency is thought to be associated with placenta underperfusion, which is a high risk factor for subsequent OS [42]. In IUGR, OS may hinder the placental neutral amino acid transport and may reduce the glucose accumulation in the syncytiotrophoblast, all of which decrease the uptake of critical materials for fetal development [43]. The relationship between IUGR and OS was reported by several authors [11, 44, 45]. In particular, neonates with IUGR showed a significant deficiency in antioxidant defenses as well as an increased lipid peroxidation [46]. F2-IsoP, which represents the main marker of the arachidonic acid peroxidation, was higher in pregnancies with fetal growth restriction and showed a moderate power on distinguish between

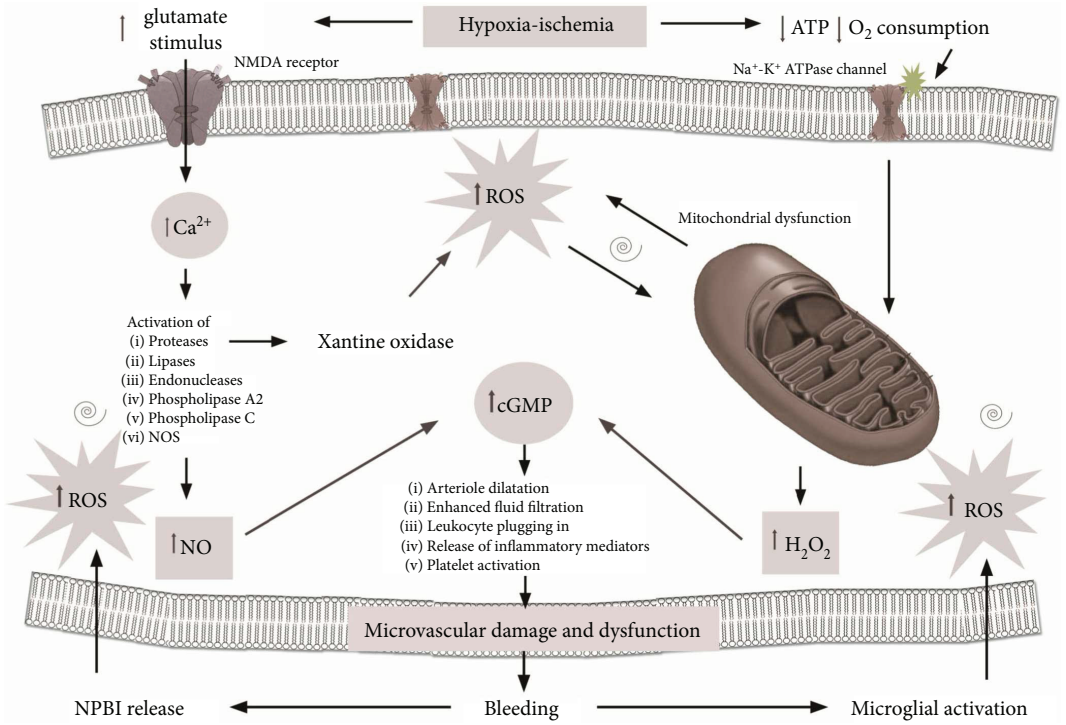

Figure 2: Schematic representation of vascular cerebral injury.

normal and restricted growth fetuses, when tested on amniotic fluid [11].

FRs may also disrupt the amino acid binding to proteins and polyunsaturated fatty acids of lipid membranes, thus causing cell dysfunction, modification of chorioamniotic biology, and, finally, the premature rupture of membranes [11]. For confirmation, higher levels of F2-IsoP were found in amniotic fluid of mothers with premature rupture of membranes, compared with control pregnancies [47].

Diabetes and obesity may induce OS in pregnancy, which in turn may cause biochemical disturbances of the fetus and newborn [48, 49]. In diabetes mellitus, ROS are produced in excess due to the prolonged periods of hyperglycemia, which is known to cause nonenzymatic glycation of plasma proteins [23]. Moreover, glucose undergoes autooxidation, thus forming FR hydroxilic anions that overwhelm the antioxidant cellular responses. The high blood glucose levels can affect the surrounding vasculature, causing the endothelium to be more sensitive to FRs [50]. To date, in diabetes experimental models, an increased ROS generation was highlighted in the embryos, fetuses, and placentas [51–53]. This increase in intrauterine OS during embryonic and feto-placental development has been associated with an impaired organogenesis [54]. A detrimental state of OS may be inherited at birth by the intrauterine environment.

3. Free Radical-Related Diseases of the Newborn: Overview

Newborns are particularly prone to OS due to the exposure to conditions that can cause a burden of FRs. Preterms have weak antioxidant defenses that are not able to counteract the harmful effects of FRs. Moreover, frequent condition such as ischemia, hypoxia-reperfusion, infection, and inflammation are triggers capable to produce high levels of FRs, thus perturbing the normal redox balance and shifting cells into a state of OS [55]. Blood transfusions, increased levels of nonprotein-bound iron (NPBI), xenobiotics, drugs, and hyperoxia are other potential sources of FR generation.

Cellular, tissue, and organ damages, involving kidney, retina, lung, and bowel injuries, have been related with OS biomarker levels in cord blood [27], thus leading to the hypothesis of free radical-related diseases of prematurity. Intraventricular hemorrhage, retinopathy of prematurity, bronchopulmonary dysplasia, and necrotizing enterocolitis have been already included in this group of conditions [26]. Later in this work, the last evidences supporting the relationship between OS and diseases of prematurity will be updated. Moreover, other conditions such as kidney damage and hemolysis will be reviewed and discussed.

3.1. Intraventricular Hemorrhage. Intraventricular hemorrhage (IVH) in very preterm infants is a common disease associated with long-term consequences [56]. The hemorrhage typically involves the periventricular germinal matrix (GM). Pathogenesis of GMH-IVH is multifactorial, complex, and heterogeneous. An inherent fragility of the GM vasculature predisposes to hemorrhage, and fluctuation in the cerebral blood flow induces the rupture of blood vessels (Figure 2). Platelet or coagulation disorders might accentuate and perpetuate the hemorrhage [57]. The inherent fragility of the germinal matrix vasculature may be further exacerbated following hypoxia, but the precise effects on cerebral blood vessels remain poorly understood [58]. Recently, more

detailed analyses have demonstrated the role of OS in this context [59]. During hypoxia, FR production increases, enhancing all the pathways implicated in microvascular damage and dysfunction. H_2O_2 and nitric oxide radicals ($NO^{\bullet}$) are able to activate the soluble enzyme guanylate cyclase, which catalyzes the formation of the cyclic "second messenger" guanosine monophosphate (cGMP). cGMP modulates the function of protein kinases, ion channels, and other important targets, leading to altered dilatation of arterioles, enhanced fluid filtration, leukocyte plugging in capillaries, and release of inflammatory mediators and platelet activation [60]. The oxidative events that trigger the initiation of bleeding into the germinal matrix promote a cascade leading to the disruption of tight junctions, to the increased blood-brain barrier permeability, and to microglial activation within the developing periventricular white matter. These events are mediated by cytokines (IL-1β and TNF-α), VEGF, and NO. Finally, reactive microglia release ROS, which in turn not only contribute to endothelial damage but also alter hemostasis and increase anaerobic metabolism [61]. In our previous study, we found increased plasma levels of total hydroperoxides (TH), advanced oxidation protein products (AOPP), and particularly NPBI in newborn who developed IVH [27]. Hypoxia and ischemia are the most important source of nonprotein-bound iron (NPBI) [62]. Moreover, the latter's conditions also supply redox-cycling iron, enhancing NPBI release into plasma [63]. Even higher increment of NPBI was described during reperfusion phase by erythrocyte releasing [64]. Due to low transferrin levels, the decreased transferrin iron-binding capacity, and low levels of ceruloplasmin and ferroxidase activities, premature infants may be particularly prone to an exaggerated generation of NPBI [65–68]. In this case, iron is capable of causing degeneration of endothelial cells [69]. Endothelial cell injury and dysfunction may additionally contribute to the inflammatory response and alteration in coagulation, through loss of normal endothelial NO production [70]. Other potential implications of iron overload are acute impairment of endothelium-dependent flow-mediated vasodilation [71], loss of tight junction proteins, degeneration of endothelial cells, and opening of the blood-brain barrier [69]. Separation of endothelial tight junctions, loss of endothelial attachment to the basement membrane, endothelial blebbing, and endothelial necrosis have been described in the cerebral vasculature following ischemic injury [72]. The progression of endothelial dysregulation can contribute to the ongoing pathogenesis of IVH.

3.2. Retinopathy of Prematurity. Retinopathy of prematurity (ROP) is the major cause of visual impairment and blindness in premature neonates worldwide [73]. ROP-associated blindness incidence has been reported to be lower than 10% of extremely preterm born children, but in some low- and middle-income countries, the incidence can reach the 40% [74, 75]. Normally, the peripheral retinal vascularization keeps developing until the fetus is near to full-term. ROP occurs in two phases: the vascular attenuation phase (phase I) and the fibrovascular proliferative phase (phase II). In phase I, a cessation of normal retinal vascularization is driven by hyperoxia, while in phase II, hypoxia renews vascularization. In both of these cases, VEGF plays a major role but in an opposite manner [76]. During hyperoxic phase I, VEGF is suppressed arresting the normal retinal vascularization and leading to the loss of some developing vessels. Later in retinal development, the oxygen need increases and a hypoxic condition arises. VEGF production begins in response to this hypoxia, thus giving rise to retinal neovascularization in the outer border, between vascularized and nonvascularized retinas [77]. The action of VEGF depends on insulin-like growth factor-1 (IGF-1) [78]. Fetal IGF-1 precipitously falls after premature birth and increases due to the newborn's maturation, thus contributing to the later neovascularization process.

It is not clear why some babies develop severe ROP whereas other babies with similar clinical characteristics do not progress to a severe stage. Genetic factors in addition to prematurity or environmental conditions may be responsible of the development and progression of ROP.

OS may represent a key mechanism in this different individual response, depending from each own redox state. Different from VEGF, OS acts in a continuum manner into ROP pathophysiology through intracellular ROS generation, which acts as signaling effectors. Several experimental studies have deepened our understanding of the role of oxidative and nitrosative compounds on ROP. In these studies, crosstalk among inflammatory, metabolic, and angiogenic pathways showed a trigger effect on pathologic or physiologic intracellular oxidative signaling mechanisms [79].

During phase I, oxygen and overall hyperoxia are fundamental factors, with a direct relationship between a high-oxygen saturation and ROP [77]. Nitrosative stress through $ONOO^-$ generation may lead to hyperoxia-induced apoptosis [78] which was found to be implicated in neuroglial injury [79] and vaso-obliteration in "oxygen-induced retinopathy" (OIR) models [80]. Transcription factor, Nrf2, showed an antioxidant protection against hyperoxia-induced endothelial loss in OIR and increases avascular retina [81].

In phase II OIR models, hypoxia- or oxidative-induced factors mediated the overexpression of VEGF signaling through VEGF receptor 2 (VEGF2). Overactive VEGFR2 then triggered downstream signaling events that disoriented endothelial cell divisions and enabled them to grow outside the plane of the retina rather than within the retina [82–84]. In this context, ROS were mainly produced by Nox [85]. Various isoforms of Nox have been implicated, including Nox-1 [86], Nox-2 [87], and Nox-4 [88]. Controversially, Nox is the key enzyme in leukocytes which serves to fight invading microorganisms; hence, inhibiting Nox may have unwanted consequences. Nitric oxide (NO) can also have beneficial effects on endothelial cells as a vasodilator, but NO may be capable to generate $ONOO^-$ in the presence of high concentrations of superoxide radical. Thus, we are presented with a double-edged sword and a balance between, or a specific targeting of, oxidative/nitrosative effects.

Clinical studies supported the risk of oxygen and hyperoxia in ROP pathogenesis, thus describing how O_2

Table 1: Antioxidants and diseases: clinical trials.

Disease	Antioxidant	Outcome	References
BPD	Lutein	Possible positive association between lutein and respiratory health	Melo van Lent, Leermakers; 2016
	iNO + vitamin A	Reduced the incidence of BPD and BPD + death and improved neurocognitive outcomes at 1 year in the 500–749 g birth weight group	Gadhia, Cutter; 2014
	Recombinant human SOD	Reduced early pulmonary injury, resulting in improved clinical status at 1 year corrected age Multiple intratracheal doses of rhSOD increase the concentration and activity of the enzyme in serum, tracheal aspirate, and urine	Davis, Parad; 2003 Davis, Rosenfeld; 1997
	Melatonin	Newborns who developed BPD had levels of IL-6, IL-8, TNF-alpha, and nitrite/nitrate values much higher than those in children who did not develop BPD	Gitto et al.; Pineal Res; 2004
	Vitamin A	Vitamin A supplementation does not reduce the incidence of BPD	Gawronski CA, Gawronski KM, Ann Pharmacother; 2016
NEC	L-Arginine	Enteral L-arginine supplementation appears to reduce the incidence of stage III NEC in VLBW infant	Polycarpou et al., 2013
	Pentoxifylline	Pentoxifylline did not change the risk of development of NEC in neonates with sepsis	Pammi et al., 2015
	Melatonin	Melatonin administration as an adjuvant therapy in neonatal NEC treatment is associated with improvement of clinical and laboratory outcome	[130]
ROP	Vitamin A	A trend towards reduced incidence of retinopathy of prematurity in vitamin A-supplemented infants	Darlow et al., Cochrane Database Syst Rev; 2002
	Vitamin E	There was a 52% reduction in the incidence of stage 3+ ROP in VLBW infants In VLBW infants, vitamin E supplementation significantly increased the risk of sepsis and reduced the risk of severe retinopathy and blindness	[94]; Brion et al., Cochrane Database Syst Rev; 2003
	D-Penicillamine	Six of the 70 surviving control infants and none of the 71 surviving treated infants had ROP stage II or greater Prophylactic enterally administered DPA does not prevent any stage ROP	Lakatos et al., Acta Paediatr; 1986 Tandon et al., Acta Paediatr; 2010
	Recombinant human SOD	rhSOD reduces the risk of developing ROP in extremely low gestational age newborn	Parad et al., Neonatology; 2012
	Lutein	There were no differences in the incidence of ROP at any stage between groups No significant effect was seen on threshold ROP	Romagnoli et al., J Matern Fetal Neonatal Med.; 2011 Manzoni et al., Am J Perinatol; 2013
	Allopurinol	Failure of allopurinol prophylaxis to prevent ROP	Russel et al., Arch Dis Child Fetal Neonatal Ed; 1995

administration in delivery room was significantly associated with the development of ROP [89]. Very high concentrations of hypoxanthine, which is produced during hypoxia/reperfusion, were found in the eyes of babies at risk of developing ROP [90].

It has been shown that the retinal antioxidant and chelator enzyme content is low in ROP cases [91]. Enhancing endogenous or exogenous antioxidant power may help in counteracting OS-related injury [92, 93]. Protective effects have been shown by giving the potent antioxidant D-penicillamine and vitamin E [94], but several further studies are needed to properly analyze other promising antioxidant agents (Table 1).

3.3. Bronchopulmonary Dysplasia. Bronchopulmonary dysplasia (BPD) is the most common adverse respiratory outcome in very preterm neonates [95, 96]. Originally, "old" BPD was defined based on lung injury occurring in premature infants born between 29 and 32 weeks of postmenstrual age (PMA), due to their respiratory distress syndrome (RDS) requiring oxygen supplementation and especially mechanical ventilation [97]. Later, the introduction of maternal corticosteroids [98] and surfactant replacement therapy [99] resulted in a different disease phenotype ("new" BPD) that was seen in younger preterm infants (below 29 weeks PMA), based on impaired alveolar and capillary development of the immature lungs [96]. Depending on the cohort and definition used, the overall incidence variates between 5 and 68% and increases significantly with declining gestational age [100]. Prematurity, oxygen toxicity, inflammation, mechanical ventilation, and surfactant deficit are major factors contributing to the pathogenesis [101]. Recently, much more importance has given to prenatal environment [102]. In example, preeclampsia alone has been defined as an independent risk factor for the subsequent development of BPD [103]. The generation of FR is one

common pathway shared by these insults. Moreover, inadequate nutrition and how the baby is ventilated participate to the increase of OS [104].

"Old" BPD is characterized by a tissue remodeling process that has been divided into different phases, ending up in the chronic phase with an increased number of fibroblasts and fibrotic areas. Matrix metalloproteinases (MMPs) are important in fibrotic processes, and the balance between MMPs and their inhibitors normally drives the development of fibrosis. MMP expression is regulated at the transcriptional level by cytokines, growth factors, and extracellular matrix components. OS increases both MMPs and their inhibitors, thus causing a disruption of the extracellular matrix [104].

The "new" BPD was observed to have less fibrotic component and a more delayed alveolar development than the older counterpart [105]. Many authors suggested that an ongoing inflammatory process could be the prior mechanism in this "atypical" BPD [101, 106]. The release of inflammatory mediators can stimulate the endothelium to produce adhesion molecules, resulting in transendothelial cytokine migration [107]. The increased cytokine concentration could therefore enter in a "final common pathway" leading to OS-related lung damage, whether triggered by infection (antenatal or postnatal) or by lung stretching (airways, alveoli, basement membrane, and pulmonary capillary endothelium) [106]. Supporting this theory, there was an increased concentration of TNF-α and other proinflammatory cytokines in tracheal secretions of mechanical ventilated newborns with BPD. Phagocyte number and interleukin concentrations were also found to be elevated in bronchoalveolar lavage fluid of infants with BPD [108]. The phagocytic cells in the lung mediate their antimicrobial functions through the release of lysozymes, peroxidases, and proteases, but in addition, ROS and NO were released. Activated neutrophils and pulmonary type II cells are also important inducers of the Fenton reaction, which lead to a greater ROS generation [105].

3.4. Necrotizing Enterocolitis. Necrotizing enterocolitis (NEC), a syndrome of intestinal ischemic necrosis, is the most common gastrointestinal emergency in preterm infants and results in significant morbidity and mortality [109, 110]. NEC has a multifactorial etiology including low gestational age, low birth weight, low Apgar scores, hyaline membrane disease, formula feeding, umbilical vessel catheterization, and intestinal ischemia. Other risk factors are the prolonged antibiotic exposure, the sensitization to cow milk proteins, the genetic polymorphism in vascular endothelial grow factor, interleukin-10, and interleukin-12 [111–114]. Among them, a common synergistic effect of OS was described [115].

When epithelial gut cells are exposed to enteral feeding, the increased metabolic OS tips the population toward apoptosis, inflammation, bacterial activation, and eventual necrosis [116]. Mitochondria are the major source of intestinal apoptotic signaling, which is activated during OS condition [117]. OS also causes the partial inactivation of cyclooxygenase-1 (COX-1) and reduces the generation of gastroprotective prostaglandins (PG) that are known to inhibit gastric acid secretion, increase mucosal blood flow, and stimulate mucus-HCO_3 secretion [118]. Ischemia and low-oxygen tension reduce the electron transport chain with subsequent excessive generation of hydroxyl radical ($^{\bullet}OH$), causing peroxidation of lipid cellular membranes and oxidative damage to proteins and other macromolecules. It is noteworthy that glutathione peroxidase (GPx), a major antioxidant enzyme in the gastric mucosa, was found to be inactivated during stress probably by excessively generated $^{\bullet}OH$ causing oxidative damage of GPx. This phenomenon seems to play a significant role in stress-induced gastric ulceration [119].

These triggering mechanisms result in significant inflammation of the intestinal tissues, release of inflammatory mediators, and downregulation of cellular growth factors. The proinflammatory cytokines activate a cascade of events leading to the eventual breakdown of the intestinal mucosal barrier and to severe NEC in some cases [120]. The immaturity of the gastrointestinal tract in preterm infants may also contribute to NEC development [121]. Toll-like receptor 4 (TLR4) expression is downregulated in the mature intestinal epithelium upon stimulation by Gram-negative lipopolysaccharide but is increased in the immature intestinal epithelium, leading to an exaggerated proinflammatory response through upregulation of the NF-κB pathway [122]. Lipopolysaccharide from Gram-negative bacteria interacts with TLR4 expressed predominantly by enterocytes. This interaction results in the breakdown of the gut barrier and allows for pathogenic bacterial translocation. A proinflammatory response follows resulting in increased production of proinflammatory cytokines (IL-6, IL-8, and TNF) as well as increased Th17 cells and a decrease in the number of T regulatory cells. The combination of these cellular responses with TLR4 signaling results in a profound inflammatory response [123, 124]. Moreover, TLR4 decreases the expression of endothelial nitric oxide synthase (eNOS), thereby reducing the formation of nitric oxide (NO), an important vasodilator. All this results in intestinal ischemia and subsequent NEC.

We found a strong association between NEC and cord blood concentrations of AOPP, NPBI, and TH, showing a clear correlation between intrauterine OS events and the risk of developing NEC [125]. Ozdemir et al. reported a significant increase of intestinal malondialdehyde (MDA) in preterm infants with NEC [126]. All-trans retinoic acid treatment reduced the intestinal MDA elevation, suggesting an active lipid peroxidation in NEC disease [126]. Consistent with these results, the administration of antioxidant drugs has been shown to reduce intestinal mucosa damaged by ischemia or inflammation [127, 128]. TNF-α and IL-1β were reduced in animal model affected by NEC and treated with melatonin [129], a highly effective antioxidant and FR scavenger. Melatonin also showed beneficial effects as an adjuvant therapy in preterm newborns [130].

All these data establish the importance of perinatal oxidative insults in injured intestinal epithelial cells, thus proving a reasonable basis for developing new interventions to interrupt those mechanisms.

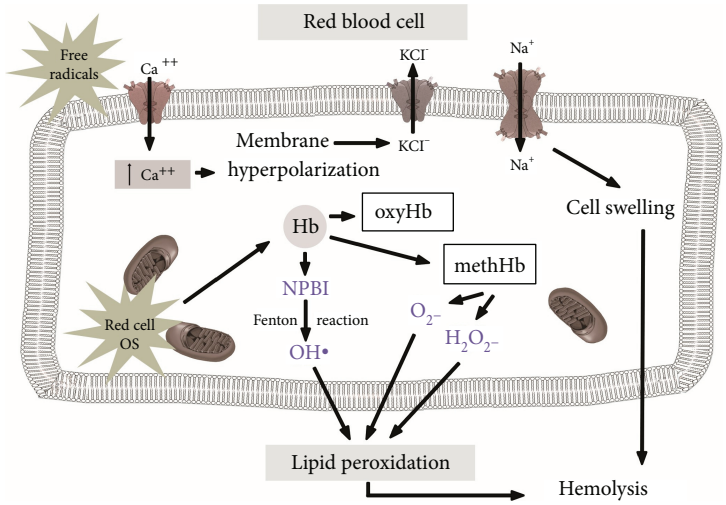

Figure 3: Schematic representation of oxidative red blood cell injury.

3.5. Renal Damage. The kidney is often severely damaged after asphyxia, but few data are available on renal oxidative damage in the newborn infant. Experimental studies show a high basal rate of aerobic metabolism in renal tissue, which suggests that the kidneys would be a primary target for this form of injury [131, 132]. The nephron of the mammalian kidney is composed of several cert types, each with distinct morphological, biochemical, and functional characteristics. One consequence of this heterogeneity is a different susceptibility to various forms of chemical and pathological injury. For example, medullary thick ascending limb cells and the pars recta of the proximal tubule are especially sensitive to injury after hypoxia or ischemia. FR-mediated lipid peroxidation has been implicated as a mechanism of tissue injury during ischemia. Lipid peroxidation products affect renal function directly by causing renal vasoconstriction or decreasing the glomerular capillary ultrafiltration coefficient and thus the glomerular filtration rate [133].

The activation of the phosphoinositide-3-kinase- (PI3K-) Akt-nuclear factor- (NF-) κB axis was detected in this rat kidney ischemia reperfusion injury model [134]. A study by Weinberg et al. on hypoxic injury to isolated renal proximal tubules showed that exogenous GSH has a protective effect [135]. Renal GSH content is decreased by hypoxia or ischemia, and the decline is most rapid immediately after the cessation of blood flow [136]. Research on the glutathione system has shown that while total GSH levels tend to decrease, GSSG levels remain constant during the progression of chronic renal failure [137], suggesting that the kidney plays an important role in the maintenance of GSH concentrations in blood. Cellular responses to OS include the induction of heme oxygenase (HO) that helps to attenuate the adverse effects of these environmental factors [138].

The induction of HO and increased ferritin synthesis may be protective in renal oxidative injury, in which increased amounts of noncatalytic-free iron have a critical role or in circumstances in which oxidative cell injury is associated with increased intracellular content of heme. The release of iron from heme proteins contributes to oxidative kidney damage through hydroxyl radical generation and lipid peroxidation [139].

Increased HO activity allows the degradation of any heme released from endogenous oxidatively denatured heme proteins, whereas increased tissue ferritin-bound iron released from heme [140]. Administration of the iron chelator desferrioxamine during reperfusion is reported to limit postischemic renal dysfunction by an effect that appears to take place in the urinary space or along the adjacent brush border membrane. Markers of renal oxidative stress have been proposed [141]. Advanced oxidative protein products seem to be a reliable marker of the degree of oxidant-mediated protein damage and the potential efficiency of antioxidant therapy. Similarly, advanced glycation end products are enhanced during renal failure and are the result of the nonenzymatic reaction linking a protein amino group with a glucose-derived aldehyde group [142].

3.6. Oxidative Hemolysis. Red blood cells (RBC) have a wide array of antioxidant enzymes defending against attacks by FRs. Superoxide dismutase (SOD), catalase (CT), and glutathione peroxidase (GPX) represent great antioxidant resources of these cells against stressors associated with prematurity [143]. Otherwise, after exposure of RBCs to OS, a rapid loss of activity of age-dependent enzymes from reticulocytes occurs probably due to proteolysis. Various experiments of hypoxia or hypoxia-reoxygenation in vitro reported increased Heinz body formation, increased oxidation products of hemoglobin, and increased intraerythrocyte hydrogen peroxide generation suggesting the increased susceptibility of red blood cells to the oxidative damage (Figure 3) [144–146]. Our extensive investigations demonstrated the key role of OS and iron release in a reactive form via the Fenton reaction in the erythrocytes. When erythrocytes were incubated in medium containing

oxidizing agents, iron release and the Fenton reaction led to the formation of the hydroxyl radical [147, 148]. Iron is released from hemoglobin or its derivatives, and the release is accompanied by methemoglobin formation. The iron diffused from erythrocytes into the incubation medium. Such diffusion, together with the higher susceptibility to release iron in newborn erythrocytes, could explain the appearance of plasma-free iron in newborns. Significant positive correlations were found between plasma-free iron and isoprostane levels in newborns, supporting the prooxidant role of free iron [149]. The higher free iron concentration, the higher lipid and protein peroxidation rate [150]. The erythrocytes are therefore a target of extracellular FR and at the same time generators of OS. The eryptosis should be considered as a cause of hemolysis when there is no evidence of an immune-mediated hemolytic anemia, no morphologic or laboratory data to suggest a problem of the red cell membrane, and no evidence of a quantitative or qualitative defect in hemoglobin synthesis in the newborns [146].

4. Conclusions

The existence of a redox homeostasis is essential for normal health and survival of the cell. When an unbalance between prooxidant and antioxidant factors occurs, OS is produced leading to cellular and tissue damage. The newborn, especially if preterm, is highly prone to OS and to the toxic effect of FRs. Taking advantage of the wealth of findings in perinatal OS researches, the relationship between OS-related mechanisms of injury and the so-called "free radical-related diseases of prematurity" can be considered irrefutable. Antioxidants may represent a very important weapon to fight this common way of damage. Collaterally, the development of biomarker OS panel has a very attractive prospect for future clinical application, in terms of prevention and response to therapy. Although much evidence already exists, more study is needed to eradicate the barrier hindering the widespread use of biomarkers. To conclude, it is no longer a long way from bench to bedside for antioxidant therapy and OS biomarkers in neonatology.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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Review Article

Oxidative Stress and Necrotizing Enterocolitis: Pathogenetic Mechanisms, Opportunities for Intervention, and Role of Human Milk

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This review will examine the role of oxidative stress (OS) in the pathogenesis of necrotizing enterocolitis (NEC) and explore potential preventive and therapeutic antioxidant strategies. Preterm infants are particularly exposed to OS as a result of several perinatal stimuli and constitutive defective antioxidant defenses. For this reason, OS damage represents a contributing factor to several complications of prematurity, including necrotizing enterocolitis (NEC). Being NEC a multifactorial disease, OS may act as downstream component of the pathogenetic cascade. To counteract OS in preterm infants with NEC, several antioxidant strategies have been proposed and different antioxidant compounds have been experimented. It is well known that human milk (HM) is an important source of antioxidants. At the same time, the role of an exclusive HM diet is well recognized in the prevention of NEC. However, donor HM (DHM) processing may impair antioxidant properties. As DHM is becoming a common nutritional intervention for high risk PI, the antioxidant status of preterm and DHM and potential ways to preserve its antioxidant capacity may merit further investigation.

1. Introduction

Necrotizing enterocolitis (NEC) is the most devastating gastrointestinal neonatal disease [1]. NEC occurs at a frequency of 1–3 per 1000 live birth, and almost 90% of the cases affect infants born preterm, the risk being inversely related to birth weight and gestational age. Despite advances in perinatal and neonatal care, NEC remains a leading cause of morbidity and mortality in preterm infants, with mortality rates reaching approximately 30% [2].

NEC is a multifactorial disease with a poorly understood pathogenesis. Prematurity itself is recognized as the main risk factor; intestinal immaturity, imbalanced microvascular tone, abnormal microbial intestinal colonization, and dysregulated immune response, together with hypoxic-ischemic injury, lead to intestinal inflammation and necrosis [3]. Due to NEC multifactorial nature and frequently abrupt onset, univocal treatment and management of the disease are still debated.

Newborns and especially preterm infants are more exposed to oxidative stress (OS) than adults and children. Reactive oxygen species (ROS) damage seems to play a role in many neonatal diseases. In 1988, Saugstad proposed the concept of "oxygen radical diseases of neonatology," including respiratory distress syndrome, bronchopulmonary dysplasia, periventricular leukomalacia, patency of the ductus arteriosus, retinopathy of prematurity (ROP), and NEC.

This article will review the role of OS in the pathogenesis of NEC and will explore potential preventive and therapeutic antioxidant strategies, with a focus on strategies for reducing OS by implementing human milk (HM) feeding.

A narrative review of published studies reporting the role of OS in the pathogenesis of NEC was performed. The literature search was run in PubMed and Embase.

The PubMed string was built up by combining all the terms related to OS and NEC, using PubMed MeSH terms, free-text words, and their combinations through the most proper Boolean operators, in order to be as comprehensive as possible. The same criteria were used for searching Embase. An additional search was performed by including HM among search criteria. The reference lists of relevant studies were searched for additional papers to be included.

2. Oxidative Stress in Neonatology

ROS and nitrogen reactive species (RNS), which are collectively referred to as ROS, are normally produced in living organisms. Biologically, significant ROS elements include hydroxyl ($OH^{\bullet}$), superoxide ($O^{-2\bullet}$), nitric oxide ($NO^{\bullet}$), hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), organic peroxides (ROOH), and peroxynitrite ($ONOO^-$). Reactive iron species are also included into ROS [4]. When produced in physiological concentrations, ROS behave as important mediators of almost all cellular functions, such as energy production and immune response. When produced in excess, they induce OS, which is responsible for tissue and cellular injury consisting in peroxidation of membrane lipids, alterations in protein function and structure, and DNA damaging.

ROS are generated via various mechanisms such as hypoxia, ischemia-reperfusion, hyperoxygenation, neutrophil and macrophage activation, mitochondrial dysfunction, endothelial cellular damage, and prostaglandin metabolism. ROS are neutralized by antioxidant systems which include endogenous and exogenous, enzymatic and nonenzymatic, and vitamin and nonvitamin components. Currently, known endogenous antioxidants include superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase, glutathione (GSH), flavonoids, bilirubin, uric acid, melatonin, endogenous organic selenium, and the metal-binding proteins transferrin, ferritin, lactoferrin, ceruloplasmin, and albumin. Exogenous antioxidants include vitamin C, vitamin E, carotenoids, phenolic acids, flavonoids, acetylcysteine, exogenous selenium, zinc, magnesium, and copper.

There is a delicate balance between free radicals and antioxidants. OS occurs when ROS production overcomes antioxidant capacity or alternatively, when the antioxidant system is defective. OS is involved in several natural phenomena, from birth to aging, starting from pregnancy and continuing during postnatal life [5]. Birth represents a significant oxidative challenge, because of the shift from the relative low-oxygen intrauterine environment to the high-oxygen extrauterine environment [6]. The fetal antioxidant system is upregulated in the last 15% of gestation in preparation for the transition to extrauterine life. During this critical time window, nonenzymatic antioxidants cross the placenta in increasing concentrations and endogenous fetal antioxidant enzyme, such as SOD, has been shown to increase by 150% [7]. Thus, newborns are exposed to OS during transition to neonatal life and labor; several studies have documented high levels of OS markers in mothers and healthy term infants immediately after birth, further increasing during the first few days of neonatal life [8].

Human studies have demonstrated that increased ROS production occurs in preterm infants compared to term infants and is associated with a relative lack of antioxidant enzyme concentration and activity. Furthermore, Sandal et al. [9] have shown that in preterm infants and term small-for-gestational age (SGA) infants, total oxidant stress measured on cord blood is higher and total antioxidant capacity is lower than in term appropriate-for-gestational age (AGA) infants.

Preterm infants are especially exposed to ROS damage for several reasons. Birth oxidative challenge may be even more difficult for preterm infants, because preterm birth interrupts the normal developmental upregulation of the antioxidant system. Moreover, the ability of preterm infants to increase antioxidant production in response to oxidant stimuli is impaired [7]. On the other hand, increased ROS production occurs in preterm infants; actually, early after birth they are often exposed to high oxygen concentration due to resuscitation and O_2 supplementation. In addition, they have particularly high metabolic turnover rates and are more susceptible to infection, because of hospitalization and relative immunodeficiency. PI also have higher levels of free iron than term infants, which can lead to the production of the highly toxic $OH^{\bullet}$ [4, 6]. Last but not least, nutritional factors contribute to ROS exposure. In preterm infants who cannot receive enteral feeding, total parenteral nutrition (TPN) is critical to provide essential nutrients. However, TPN solutions are often contaminated with oxidation product [10]. Even when enteral nutrition is introduced, mother milk availability is often low and formula milk is used instead. HM is known to be a better scavenger of free radicals than infant formula [11]. Studies have demonstrated that dietary factors may contribute to the overall level of oxidative stress in preterm infants; specifically, preterm infants fed with HM have shown less evidence of oxidative stress after birth than those who were formula fed [12].

Normal pregnancy is associated with enhanced ROS production due to a high metabolic rate and oxygen requirements. The maintenance of pregnancy and fetal development depends on oxygen supply and ROS neutralization; moreover, in complicated pregnancy OS increases. Whenever OS occurs, it may cause abnormal placentation, endothelial dysfunction, and abnormal fetal growth, and it has been associated to several pregnancy disorders including preeclampsia, fetal growth restriction, and preterm birth [5]. OS markers have been measured in cord blood of preterm infants as an indicator of perinatal OS. They have been associated with increased risk for "free radical-related diseases," suggesting that in utero/perinatal OS is a significant risk factor, especially in premature newborns [13].

Several investigators examined the relationship between the oxidative state of the mother and the newborn at birth. A positive correlation was found between the oxidative status of healthy mothers and that of term infants measured in venous maternal and umbilical cord blood, respectively, with higher maternal OS correlating with an even higher OS of the

newborn [14]. Other studies have investigated the relation of different OS markers and lipid-soluble micronutrients, in cord plasma and maternal serum, with growth retardation and prematurity, showing contrasting results [15].

3. The Role of Oxidative Stress in the Pathogenesis of NEC

Several studies have suggested a role of OS in the pathogenesis of NEC; Aydemir et al. [16] compared, in preterm infants with and without NEC, the global oxidant/antioxidant status, by measuring total antioxidant capacity (TAC), total oxidant status (TOS), and oxidative stress index (OSI). Infants with NEC had significantly higher TOS and OSI levels compared with controls, higher levels of TOS and OSI being associated with the severity of NEC. Furthermore, Perrone et al. [17] demonstrated a strong association between the concentration of OS markers in cord blood and the occurrence of NEC in preterm infants.

According to epidemiologic observations and studies performed in animal models, the pathogenesis of NEC is thought to be multifactorial and largely related to the immaturity of the gastrointestinal tract [3]. The intestinal mucosa of preterm infants is exposed to constant injury, due to perinatal insults such as hypoxia, hypothermia, and formula feeding. Innate immune receptor Toll-like receptor 4 (TLR4) seems to have a central role in NEC pathogenesis. Excessive signaling in the epithelial TLR4 pathway in response to lipopolysaccharide (LPS) presented by Gram-negative bacteria leads to the loss of enterocytes through apoptosis, followed by delayed repair through inhibition of migration and TLR4-mediated loss of intestinal stem cells [18]. These factors lead to the translocation of bacteria and LPS into the circulation and consequently to proinflammatory cytokine production, ROS production, increased expression of inducible nitric oxide synthase (iNOS), and impaired perfusion via loss and dysregulation of endothelial nitric oxide synthase (eNOS) mediated by TLR4 [19].

Experimental models have provided clues of a direct link between ROS production in the premature gut and NEC. OS may act as a downstream component in the inflammatory cascade which results in intestinal injury and even as a concomitant cause. Nitric oxide seems to play a crucial and ambiguous role in OS-related NEC damage. NO is produced from arginine in a reaction catalyzed in the intestine mainly by two NO synthases (NOS), eNOS and iNOS. eNOS is constitutively expressed in the intestinal capillaries, and low concentrations of NO are responsible for the regulation of vascular tone. iNOS is mainly located in immune cells and activated by proinflammatory cytokines during inflammation and pathogen response. Sustained upregulation of iNOS in the intestinal mucosa is known to occur in preterm infants during the development of NEC [20]. In a neonatal rat model of NEC, increased concentration of iNOS caused by LPS was found in the intestinal mesentery in the late stage of the disease [21]. This upregulation may contribute to intestinal injury via high levels of NO. NO readily reacts with the $O^{-2\bullet}$ to form peroxynitrite, a reactive oxygen and nitrogen species that is highly toxic to epithelial cells [22]. eNOS is involved as well [23]. During OS, eNOS switches from producing NO to $O^{-2\bullet}$ [24]. This switch in enzyme function is called "eNOS uncoupling." Once generated, $O^{-2\bullet}$ can form additional ROS, exaggerating the uncoupling. Studies on rat models indicate that NOS uncoupling becomes worse during disease progression [21].

It has also been proposed that the underlying initial condition in NEC pathogenesis is the reduced ability of the neonatal gut epithelial cells (NGECs) to clear OS when exposed to enteral feeding [25]. An agent-based computational model has demonstrated that impaired OS management can lead to apoptosis and inflammation of NGECs when additional bacterial TLR4 activation occurs.

4. Potential Antioxidant Strategies for Preventing NEC

Given the role of OS in the pathogenesis of NEC and the relatively deficient oxidant/antioxidant balance in preterm infants, several studies have investigated the potential protective role of direct and indirect antioxidant strategies; recently, astragaloside IV (AS-IV), a flavonoid, has been found to be effective in the protection of NEC-induced ileum degeneration via the regulation of the vitamin D3-upregulated protein 1/nuclear factor- (NF-) κB signaling pathway [26]. AS-IV has also been shown to decrease the levels of malonyldialdehyde (MDA), one of the products of lipid oxidation, and to improve the levels of GSH and SOD in rats with experimental NEC. In addition, AS-IV inhibited NEC-induced elevation of proinflammatory mediators, such as interleukin- (IL-) 6, IL-1β, tumor necrosis factor- (TNF-) α, and NF-κB. Similarly, Ozdemir et al. [27] have suggested that all-*trans*-retinoic acid (ATRA), an active and natural derivative of vitamin A, has a protective effect on intestinal injury through its anti-inflammatory and antioxidant properties. Rats with NEC injected with ATRA showed lower intestinal MDA and TNF-α levels and higher SOD and glutathione peroxidase activities than controls. Yazc et al. [28] have found that boric acid and 2-aminoethoxydiphenyl borate partly prevent the occurrence of NEC, by decreasing GSH consumption and enhancing the antioxidant defense mechanism. Sheng et al. [29] have shown that hydrogen-rich saline reduced the incidence and severity of NEC in a neonatal rat model, by inhibiting mRNA expression of proinflammatory mediators, downregulating lipid peroxidation, and enhancing total antioxidant capacity. According to Ozdemir et al. [30], N-acetylcysteine (NAC) also has a protective effect on intestinal injury, through its anti-inflammatory and antioxidant properties. NEC rats treated with NAC had lower intestinal MDA levels and higher antioxidant enzyme activity; NAC also reduced intestinal TNF-α concentration. In vitro studies [31] suggested that hydrogen sulfide successfully rescues epithelial cell damage induced by OS in rat intestinal cells. Karatepe et al. [32] showed that the overexpression of the vascular endothelial growth factor in an experimental NEC model enhanced angiogenesis, alleviated villous atrophy and tissue edema, and was linked to reduced inflammation, apoptosis, and OS markers.

5. Exploring the "Milky Way": Reducing Oxidative Stress by Implementing HM Feeding

It is well known that the characteristics of enteral feeding have a strong impact on the risk of NEC in preterm infants; specifically, it is generally recognized that the risk of NEC is reduced when preterm infants are fed an exclusively HM-based diet compared to diets containing bovine-derived products (formula and/or traditional HM fortifiers) [33]; the detrimental effect of bovine-based products appears to be related to an increased intestinal permeability, a direct toxic effect to the gut epithelial cells, and also to an upregulation of OS [34].

Friel et al. [35] demonstrated that HM, provided by mothers of both term and preterm infants, has better antioxidant properties than formula and that preterm and term HM have equal resistance to OS. The same authors also examined the effect of HM fortification on markers of OS in preterm infants and found that infants fed with HM plus HM fortifier had the highest urinary levels of F2-isoprostanes, compared to both infants fed with exclusive HM and formula [12]. This finding, which has not been further explored, might explain, at least partially, the beneficial effect of HM diets without any bovine-derived supplement on NEC incidence in preterm infants. Sandal et al. [9] investigated antioxidant properties of preterm and term HM, showing no difference in milk oxidant status between mothers of term and preterm infants. According to these data and given the relationship between OS and NEC, ad hoc strategies aimed at implementing exclusive HM feeding in infants at risk for NEC appear to be of paramount importance.

When own mother's milk (OMM) is unavailable, donor HM (DHM) provided by a HM bank is considered the best alternative for feeding preterm infants [36], and recent data have suggested that DHM feeding is linked to a beneficial effect in terms of NEC reduction [37].

Preparation, pasteurization, and distribution of DHM are regulated by specific recommendations [38–41]. The Holder pasteurization method is universally recommended for HM banks, as it guarantees the best compromise between microbiological safety and preservation of HM bioactive components [42]. Pasteurization is known to impair several nutritional and functional components of HM, such as immunoglobulin and lactoferrin [43]. Furthermore, Holder pasteurization, which is the pasteurization method commonly used in HM banks, appears to impact on the antioxidant capacity of DHM [44, 45] and to affect also selected antioxidant compounds which are present in HM, including GSH [46] and vitamin D [47]. Hanson et al. [11] recently compared several antioxidant components, including α-carotene, β-carotene, lycopene, lutein + zeaxanthin, retinol, and α- and γ-tocopherol, between preterm and donor HM; the authors documented lower levels of all the examined components in DHM compared to those in preterm HM.

The use of DHM has become common practice in many neonatal units, when OMM is insufficient or unavailable. However, since experimental data suggest that DHM processing negatively affects many functional properties of HM, it seems reasonable to explore novel treatments for DHM, aimed at preserving as many functional properties as possible [38], including antioxidant components [48]. In this perspective, growing literature is exploring the role of pasteurization methods alternative to Holder in preserving HM bioactive components, such as functional proteins [49] and fatty acids [50]. However, at present, no data about the effect of pasteurization methods other than Holder on OS markers are available.

For this reason, further research should be targeted to specifically explore the effect of DHM processing on HM antioxidant status. Whether HM antioxidant properties would result to be impaired by HM processing, it would be reasonable to explore if exogenous supplementation of antioxidant compounds would help in restoring these properties, thus preventing NEC occurrence or reducing NEC severity. In this perspective, current literature about specific targets for further research is now explored.

5.1. Glutamine and Arginine. In an experimental neonatal rat model of NEC, enteral supplementation with glutamine and arginine has been shown to exert a favorable effect on lipid peroxidation and antioxidant enzyme levels in the small intestine [51], probably through an increased production of NO. A recent systematic review of published randomized controlled trials (RCTs) [52] suggested L-arginine supplementation to be protective against NEC without any effect on neurodevelopmental outcomes at 3 years correct age. Although promising, these results were based on data from only two trials including 235 preterm infants, thus needing to be confirmed on a larger scale.

5.2. Carotenoids. As previously shown, carotenoids are antioxidant compounds which are present in preterm HM and whose concentration is severely impaired by pasteurization [11]. A multicenter RCT was conducted in order to examine the role of lutein and zeaxanthin enteral supplementation on the incidence of OS-related diseases in preterm infants [53]; carotenoid supplementation was related with a nonsignificant decrease in the incidence of NEC and ROP, and with a 50% decrease in the rate of progression from early to threshold ROP.

5.3. Melatonin. Melatonin is excreted in term and preterm HM in similar amounts; interestingly, melatonin, similarly to other antioxidant compounds, is excreted in preterm HM following a circadian rhythm [54]. Several studies have focused on the role of melatonin in counteracting oxidative injury in newborns; melatonin has been used in cases of asphyxia, respiratory distress syndrome, and sepsis, with no report of any significant side effect [5]. Data about the potential use of melatonin for NEC are limited to neonatal rat models; in rats with experimental NEC who did not receive melatonin, MDA and protein carbonyl content were higher and SOD and glutathione peroxidase levels were lower than in controls. On the contrary, rats with NEC who were also treated with melatonin had an OS profile similar to

controls, suggesting a potential role of melatonin for reducing the severity of NEC [55].

5.4. Human Milk Oligosaccharides. HM oligosaccharides (HMO) have been shown to exert a series of beneficial effect on the neonatal gut [56]. Good et al. evaluated the role of 2′-fucosyllactose (HMO-2′FL), an abundant HMO, for the protection against NEC in rats. Their data showed that the addition of HMO-2′FL to milk formula reduced the severity of NEC. HMO-2′FL protective effects occurred via restoration of intestinal perfusion through upregulation of eNOS and downregulation of proinflammatory molecules including iNOS [57]. Thus, HMO may have indirect antioxidant properties by modulating the NO pathway. Interestingly, HMO do not seem to be affected by Holder pasteurization [43].

5.5. Lactoferrin. Lactoferrin plays a role in iron homeostasis, anti-inflammation, and host defense against microbial infections. Recently, it has been shown that lactoferrin inhibits the production of intracellular ROS in human mesenchymal stem cells, which suggests a potential role of lactoferrin in the prevention of OS-related damage [58]. Several RCTs have explored the effectiveness of oral lactoferrin in the prevention of NEC in preterm neonates. In a recent Cochrane review, results of six RCTs on this topic have been summarized; oral lactoferrin supplementation reduced late-onset sepsis and stage II and III NEC [59]. However, as the authors of the review also pointed out, the evidence about routine supplementation with lactoferrin is still of low quality and optimal dosing regimen, source of lactoferrin (human or bovine), long-term outcomes, and mechanisms are still to be clarified.

5.6. MicroRNAs. It has been recently documented that microRNAs (miRNAs) are present in HM and that specific miRNAs are involved in the innate and acquired immune response. Apparently, miRNA distribution and expression profile are not affected by pasteurization [60]. As for NEC, Ng et al. explored the role of dysregulated miRNAs in the pathogenesis of NEC and spontaneous intestinal perforation in preterm infants. According to the results of the study, dysregulation of miRNA/mRNA pairs played a significant role in the disease pathogenesis, with mechanisms also involving OS-related pathways [61]. This data warrant further exploration of HM miRNA characteristics and relation with neonatal gastrointestinal diseases.

5.7. Gut Microbiota. The role of intestinal dysbiosis is emerging as a major pathogenetic factor for NEC [62], and probiotics have been suggested as a promising intervention for preventing NEC in preterm infants [63]. It has been demonstrated that fecal microbiota transplantation (FMT) is effective in a mouse model of NEC through OS modulation and reduced TLR4-mediated colonic inflammation [23]. FMT eliminated superoxide production and promoted NO production, contrasting eNOS uncoupling. Furthermore, Ferretti et al. [20] identified iNOS as a potential mediator of the effects of both epithelial growth factor and indomethacin, which were, respectively, associated to positive and detrimental effects on the immature human gut. Their findings further indicate that the modulation of the NOS-NO pathway may represent a therapeutic opportunity for NEC.

6. Conclusions

NEC represents a clinical and research priority; a better understanding of NEC pathogenetic pathways may offer new treatment perspectives. OS has been shown to play a role in NEC cascade, and several antioxidant strategies have been explored. Despite promising preclinical studies, more clinical trials are needed. To date, the role of an exclusive HM diet is well recognized in the prevention of NEC. At the same time, it is known that DHM processing severely impairs HM functional properties, including defenses against OS. For this reason, future research should be targeted to explore in detail antioxidant properties of preterm and donor HM and to evaluate the best way to preserve or restore any deficient antioxidant function.

Conflicts of Interest

None of the authors has any conflict of interest to declare in connection with this paper.

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Research Article

Early Prediction of Hypoxic-Ischemic Brain Injury by a New Panel of Biomarkers in a Population of Term Newborns

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This research paper is aimed at evaluating the predictive role of a default panel of oxidative stress (OS) biomarkers for the early identification of infants at high risk of HIE and their validation through the correlation with MRI findings. A multicenter prospective observational study was performed between March 2012 and April 2015 in two European tertiary NICUs. Eighty-four term infants at risk for HIE (pH < 7, BE < −13 mmol/L, and 5′ Apgar < 5) were enrolled. Three were excluded for chromosomal abnormalities and one due to lack of blood samples. The final population was divided according to the severity of perinatal hypoxia into 2 groups: mild/moderate HIE and severe HIE. Advanced oxidation protein products (AOPP), non-protein-bound iron (NPBI), and F2-isoprostanes (F2-IsoPs) were measured in blood samples at P1 (4–6 hours), P2 (24–72 hours), and P3 (5 days), in both groups. MRIs were scored for the severity of brain injury, using a modified Barkovich score. The mean GA was 39.8 weeks (SD 1.4) and the mean birth weight 3538 grams (SD 660); 37 were females and 43 males. Significantly lower 5′ Apgar score, pH, and BE and higher Thompson score were found in group II compared to group I at birth. Group II showed significantly higher AOPP and NPBI levels than group I (mean (SD) AOPP: 15.7 (15.5) versus 34.1 (39.2), $p = 0.033$; NPBI 1.1 (2.5) versus 3.9 (4.4), $p = 0.013$) soon after birth (P1). No differences were observed in OS biomarker levels between the two groups at P2 and P3. A regression model, including adjustment for hypothermia treatment, gender, and time after birth, showed that AOPP levels and male gender were both risk factors for higher brain damage scores (AOPP: OR 3.6, 95% CI (1.1–12.2) and gender: OR 5.6, 95% CI (1.2–25.7), resp.). Newborns with severe asphyxia showed higher OS than those with mild asphyxia at birth. AOPP are significantly associated with the severity of brain injury assessed by MRI, especially in males.

1. Introduction

Birth asphyxia is largely recognized as the most frequent cause of acute interruption of oxygen to the fetus and the most common cause of brain damage [1]. Currently, despite the advances offered by therapeutic hypothermia in terms of neuroprotection, the improvements on long-term neurological outcome remain modest [2–4]. Twenty to fifty percent of asphyxiated infants who develop HIE die in the neonatal period, and about twenty-five percent of survivors will develop neurological disabilities, such as cerebral palsy, cognitive deficits, learning disorders, sensory disruption, and neuropsychiatric problems [5]. Therefore, one of the most important goals in the approach to patients with HIE remains actually to determine the exact period in which the effects of potential damaging factors occur [1, 2, 5, 6]. Several methods are now available for detecting the type and timing of brain damage: conventional prenatal tests, such as fetal

cardiotocography; ultrasound; Doppler and amniotic fluid examination neuroimaging; aEEG; NIRS; and determination of numerous currently available biomarkers. Each provides information about different expressions of brain injury and has some limitations [1, 7]. MRI is the gold standard for the early evaluation of brain injury after HIE, including not only traditional neuroimaging methods but also advanced imaging techniques (DWI, ^{1}H-MRS, and ASL) [8–11]. In this context, the use of specific biomarkers that will increase within the first hours of life in hypoxic-ischemic neonates may help in the early diagnosis of HIE and promptly identify neonates who may qualify for neuroprotection. Oxidative stress is involved in the mechanisms of hypoxic-ischemic and inflammatory brain injury, although the relationship between brain damage and OS is very complex and not entirely clear [12–15]. The pathophysiological process that leads to the development of brain lesions is in fact characterized by the combination of several mechanisms, either exogenous or endogenous (hypoxia, ischemia, ischemia-reperfusion, hyperoxia, inflammation, and mitochondrial damage), whose effect on cell biology and on oxidative metabolism varies according to the severity and duration of the insult [16]. Furthermore, certain brain areas are particularly rich in iron, released by cells damaged during hypoxia, which may catalyze, through the Fenton reaction, the formation of hydroxyl radicals and nitroperoxide and so make the central nervous system more susceptible to the attack of the reactive species [17]. In addition, the brain of a full-term baby, being rich in polyunsaturated fatty acids and low in antioxidants, is particularly vulnerable to the free radical attack [18]. Increased oxidative stress in hypoxic fetuses and neonates has been detected by assaying several biomarkers: products of lipid peroxidation in expired air, serum malondialdehyde reaction, serum isoprostanes, serum total hydroperoxides, advanced oxidative protein products, and increased NPBI in serum [18–20]. Despite extensive research in the field over the last few years, no such biomarker has been validated in clinical practice so far. So the aim of our study was to evaluate the predictive role of a default panel of OS biomarkers for the early identification of infants at high risk of hypoxic-ischemic brain injury and their validation through the correlation with MRI.

2. Methods

2.1. Subjects. Eighty-four term subjects, born between March 2012 and April 2015, with clinical and biochemical signs of HIE, admitted to two European tertiary NICUs as part of a multicenter prospective observational study, were consecutively enrolled. The inclusion criteria were the presence of perinatal asphyxia defined as at least three of the following criteria: (1) late decelerations on fetal monitoring or meconium staining; (2) delayed onset of respiration, resuscitation, or ventilation of at least 10 min; (3) Apgar scores < 5 at 5 minutes; (4) arterial cord blood pH < 7.1 with a base deficit > 16 mmol/L or serum lactate > 10 mmol/L; (5) multiorgan failure, followed by symptoms of encephalopathy, such as altered alertness, abnormal tone, feeding difficulties, or seizures demonstrated by a Thompson score ≥ 7 and/or abnormal brain activity by aEEG; and (6) hypothermia treatment started within 6 h after birth [4, 7, 21]. HIE was classified as mild (grade I), moderate (grade II), or severe (grade III) according to the criteria described by H.B. Sarnat and M.S. Sarnat [22]. The clinical evaluation of encephalopathy took place 24 and 48 hours after birth. Babies with major congenital abnormalities, brain malformations, central nervous system infections, and inborn errors of metabolism were excluded. As soon as possible after admission to the Neonatal Intensive Care, all enrolled children were subjected to the routine checks, including a blood gas analysis, and were started on aEEG and NIRS monitoring. Hypothermia was initiated within 6 h after birth, lasted for 72 h, and was aimed for a rectally measured body temperature of 33.5°C. Seventy-two hours after starting hypothermia, subsequent rewarming at 0.5°C per hour was performed. Body temperature (°C), heart rate, arterial blood pressure, and arterial oxygen saturation (SaO_2) were monitored simultaneously with NIRS and aEEG parameters, and their recorded values were all stored on a personal computer for off-line analysis (software: Poly 5, Inspektor Research Systems, Amsterdam, the Netherlands). All clinical and demographic data were collected from the hospital records. The study was approved by the medical ethical review board of the two respective university hospitals, with written informed parental consent, obtained according to the Declaration of Helsinki.

Table 1: Scoring system for brain injury seen on MRI scans.

Score	Description
Basal ganglia and thalamus	
0	Normal
1	Abnormal signal in the thalamus
2	Abnormal signal in the thalamus and lentiform nucleus
3	Abnormal signal in the thalamus, lentiform nucleus, and perirolandic cortex
4	More extensive involvement
Watershed areas	
0	Normal
1	Single focal infarction
2	Abnormal signal in the anterior or posterior watershed white matter
3	Abnormal signal in the anterior or posterior watershed cortex and white matter
4	Abnormal signal in both anterior and posterior watershed zones
5	More extensive cortical involvement
Posterior limb of the internal capsule	
0	Myelination present
1	Myelination present but impaired
2	Myelination absent

2.2. Oxidative Stress Methodology. Advanced oxidation protein products (AOPP), F2-isoprostanes (F2-IsoPs), and non-protein-bound iron (NPBI) were all measured in blood samples, taken during routine tests and only after obtaining

Table 2: Clinical and biochemical signs of hypoxic-ischemic encephalopathy reported by groups.

	Mild-to-moderate HIE group ($n = 21$)	Severe HIE group ($n = 59$)	p
Median Apgar 1 min (IR)	2 (1–4)	1 (0–2)	NS
Median Apgar 5 min (IR)	4 (4-5)	3 (1–5)	0.002
Median Apgar 10 min (IR)	7 (5–7)	6 (3–7)	0.011
Mean umbilical pH (SD)	7.0 (0.1)	7.0 (0.2)	NS
Mean umbilical BE (mmol/L) (SD)	−13.7 (6.2)	−13.8 (8.3)	NS
Mean pH at admission (15 min–6 h of life) (SD)	7.1 (0.1)	6.9 (0.2)	0.043
Mean BE at admission (15 min–6 h of life, mmol/L) (SD)	−11.1 (6.7)	−16.3 (7.7)	0.013
Mean lactate at admission (15 min–6 h of life, mmol/L) (SD)	13.6 (5.2)	15.4 (7.8)	NS
Mean PNA at MRI (days) (SD)	3 (2)	6 (4)	NS
Median Thompson score (1 h of life) (IR)	4 (2–5)	9 (7–13)	0.000
Seizures n (%)	2 (9)	27 (54)	0.001

IR: interquartile range; SD: standard deviation. NS: not statistically significant.

the parents' written consent, at P1 (4–6 h after birth), P2 (24–72 h after birth), and P3 (5 days after birth). For each blood sample, 2.5 ml of blood was collected: 1.3 ml in EDTA (ethylenediaminetetraacetic acid) tubes and 1.2 ml in two test tubes (0.6 ml each) containing heparin. Each of these samples was immediately centrifuged (Prog 1, RTM 1500, T 4°C, 10 min) to remove cells and obtain the supernatant, which was then separated into five different microtest tubes, one of which contains BHT (butylated hydroxytoluene), and stored at −80°C. The obtained samples were subsequently analyzed to measure OS biomarkers. AOPP and F2-IsoPs were detected as markers of protein and lipid OS-induced injury, respectively, by the method of Witko-Sarsat et al., using spectrophotometry on a microplate reader, and isoprostanes were detected according to the LC-MS/MS methodology described by Casetta et al. [23, 24]. The AOPP were calibrated with chloramine-T solutions that absorb at 340 nm in the presence of potassium iodide. In test wells, 200 μL of plasma diluted at 1 : 5 in phosphate-buffered saline solution (PBS) was distributed on a 96-well microtiter plate, and 20 μL of acetic acid was added. In standard wells, 10 microliters of 1.16 M potassium iodide was added to 200 μL of chloramine-T solutions followed by 20 μL of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm on the microplate reader against a blank containing 200 μL of PBS, 10 μL of potassium iodide, and 20 μL of acetic acid. Because the absorbance of chloramine-T at 340 nm is linear up to 100 μmol/L, AOPP were expressed as μmol/L of chloramine-T equivalents. NPBI was detected as a marker of OS potential risk, by HPLC using the method described by Paffetti et al. [25].

2.3. MRI Scoring. Depending on their clinical condition, infants underwent MRI at a postnatal age of 5 ± 3 days. Intravenous sedation was continued during the MR examination for infants who had an intravenous line placed; others received an oral sedation with chloral hydrate (50–60 mg/kg). Infants were wrapped into a vacuum cushion to minimize motion, and earmuffs (EM's 4 Kids, Everton Park, Australia) were used for hearing protection. Respiratory rate (Philips Medical Systems, Best, the Netherlands), heart rate, and transcutaneous oxygen saturation (Nonin Pulse Oxymetry, Nonin Medical, Plymouth, MN) were monitored during MR imaging, and a neonatologist was present throughout the examination. The severity of brain injury was assessed by using conventional axial T1- and T2-weighted spin-echo sequences, DWI, and ADC maps. MRIs were reviewed retrospectively by two expert investigators (LV and FG) who were blinded to the infant's outcome. Injury was scored for the basal ganglia and thalami in combination with cortical involvement, the watershed areas, and the posterior limb of the internal capsule, by using the modified Barkovich score (Table 1), ranging from 0 (no damage) to 11 (massive brain damage), described previously as being predictive for neurodevelopmental outcomes after HIE [21, 22].

2.4. Statistical Analysis. Descriptive and inferential analyses were performed using the SPSS v23 for Windows statistical package (SPSS Inc., Chicago, IL, USA). Data are presented as mean and SD or median and interquartile range (IR) for descriptive analysis of continuous variables, whereas for categorical variables, the absolute frequencies are reported. A logarithmic transformation was performed for the variables that were not parametrically distributed. The independent t-test and the Mann–Whitney U test were used, where appropriate, to make comparisons regarding all patient characteristics, OS biomarker measurements at each time point, and gender differences. Pearson correlation and scatter dot plot were used, respectively, to examine and visualize the relationship between OS biomarkers and MRI score for each period of interest. A longitudinal model was built to analyze the association between OS biomarkers and the brain damage measured through the Barkovich score. Gender, treatment with hypothermia, and time of life (corresponding to the selected periods of blood sample collection) were introduced into the model as confounding factors. ROC curve was performed to find an MRI score cut-off able to discriminate between newborns with a major risk to die and newborns with a good outcome. A multivariable logistic regression model was then built to verify if the increased level of OS biomarkers may be a risk factor for

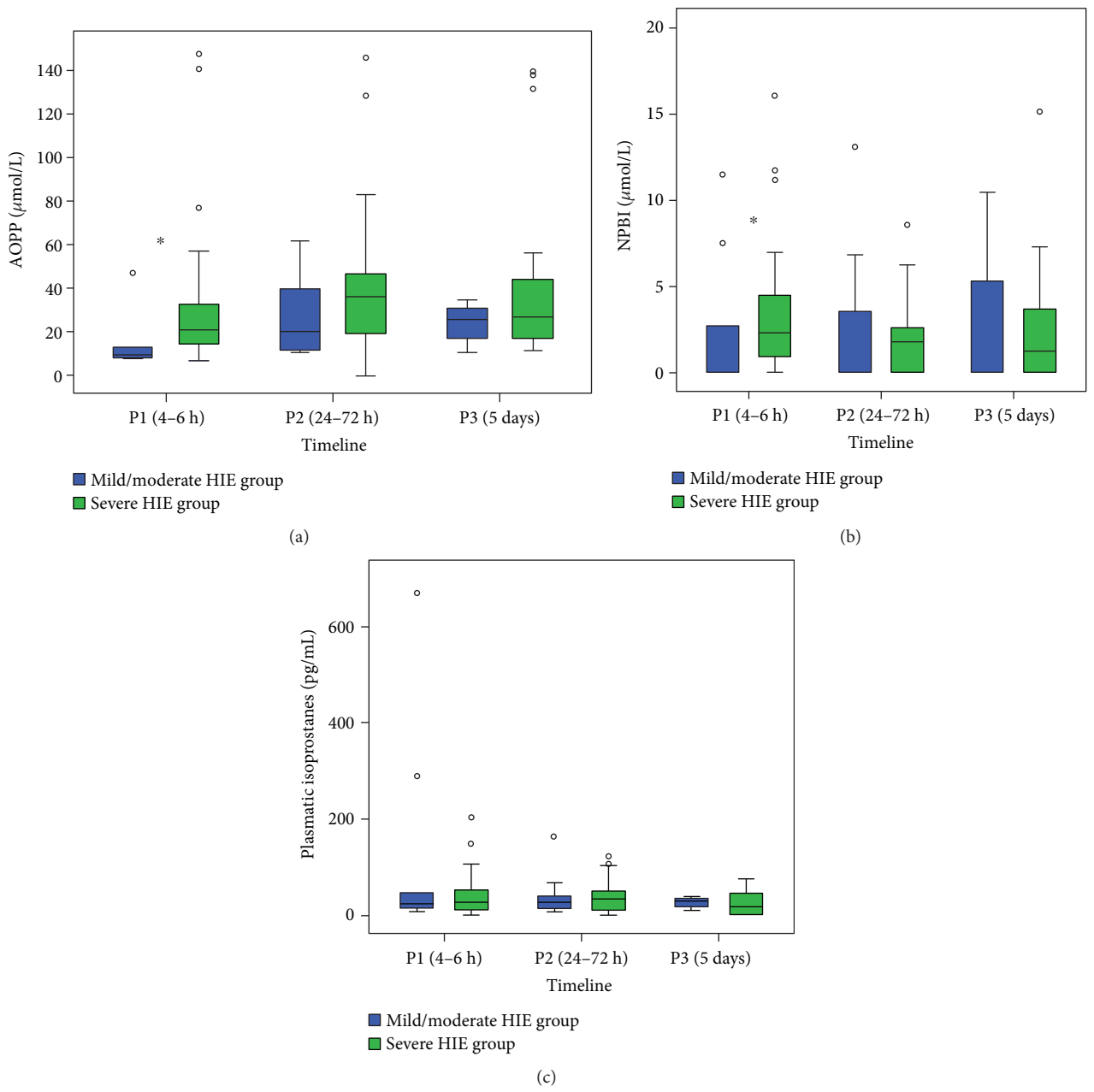

Figure 1: Relationship between OS biomarkers and the grade of perinatal hypoxia reported over time. Comparison of AOPP levels (μmol/L) (a), NPBI levels (μmol/L) (b), and IsoP levels (pg/mL) (c), between each group in the first 5 days of life. AOPP: advanced oxidation protein products; NPBI: non-protein-bound iron; ○: outliers. $^{*}p < 0.05$.

neurological damage, measured using MRI. The MRI score was introduced into the model as a dichotomic dependent variable, using the ROC curve cut-off; gender, treatment with hypothermia, and time of life were also considered in the model as covariates together with each biomarker. A p value < 0.05 was considered statistically significant.

3. Results

Out of eighty-four enrolled patients, three were excluded for chromosomal abnormalities and one due to lack of blood samples. So the final population consisted of eighty infants with a mean gestational age of 39.8 weeks (SD 1.4) and a mean birth weight of 3538 grams (SD 660); 37 were females and 43 males. Twenty newborns at risk for HIE were classified with mild (Sarnat I), 1 was classified with moderate (Sarnat II), and 59 were classified with severe signs of HIE (Sarnat III). The mild group was not considered for hypothermia, while the severe group was considered eligible for hypothermia. The moderate one was eligible for hypothermia treatment; however, he was born in a peripheral hospital and arrived too late (thus later than 6 hours after birth) to perform it. Newborns were then divided according to the severity of HIE into two groups: mild/moderate HIE (not eligible/late for the treatment with hypothermia, $n = 21$) and severe HIE (eligible for hypothermia treatment, $n = 59$).

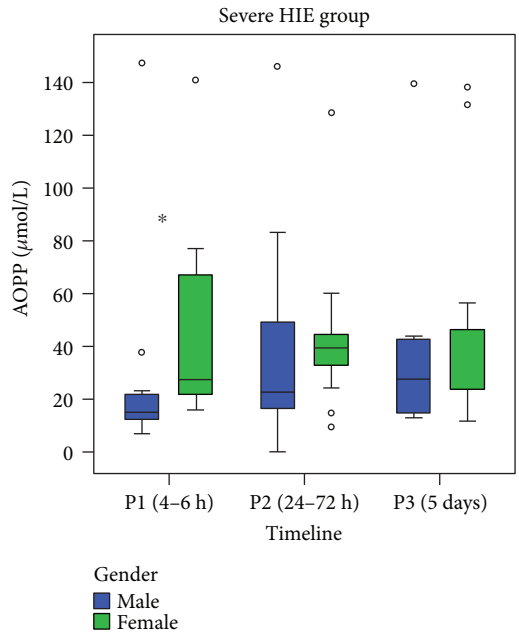

Figure 2: Relationship between AOPP levels (μmol/L) and the grade of perinatal hypoxia over time, reported by gender. AOPP: advanced oxidation protein products; ∘: outliers. $^{*}p < 0.05$.

Clinical and biochemical signs of HIE for each group are reported in Table 2. None of the infants of the mild group showed signs of progression to moderate or severe HIE.

3.1. Relationship between OS Biomarkers and the Grade of HIE—Comparison between the Two Groups over Time. The mild/moderate HIE group showed significantly lower AOPP and NPBI levels than the severe HIE group at time P1 (AOPP median (IR): 9.4 (13.6) versus 18.6 (19.5) μmol/L, $p = 0.033$ and NPBI median (IR): 0.0 (3.8) versus 2.4 (3.3) μmol/L, $p = 0.013$, resp.) (Figures 1(a) and 1(b)). No differences were observed in AOPP and NPBI levels in P2 and P3 (Figures 1(a) and 1(b)). No other differences were observed in F2-IsoP levels between the two groups (Figure 1(c)).

The severe HIE group showed also significantly lower AOPP levels in males than in females at time P1 (AOPP median (IR): 14.8 (10) versus 27.6 (50.7) μmol/L, $p = 0.013$; Figure 2). No differences in AOPP levels were observed between males and females in P2 and P3. No differences in NPBI and IsoP levels were found between males and females at any time.

3.2. MRI Score and Survival. Out of eighty enrolled patients, fifty-six (70%) underwent MRI at a postnatal age of 5 ± 3 days: respectively, 5 of 21 (23%) of the mild/moderate asphyxia group and 51 of 59 (86%) of the severe asphyxia group, according to their clinical condition. Each MRI score and the corresponding outcome are reported in Table 3.

The ROC curve discriminating newborns with a major risk to die versus newborns surviving without impairments was set at an MRI score value of 4.5 (100% sensitivity, 97.7% specificity, $p = 0.0001$; Figure 3). The MRI score ranging from 0 to 4.5 showed 100% sensitivity and 100% of true negative fraction for a good neurological outcome. Conversely, 100% of poor neurodevelopmental outcome was observed for MRI score values > 8.5. The MRI score plotted curve indicated 4.5 as the best predictive threshold with a sensitivity of 100% (95% CI 63.06–100) and a specificity of 97.7% (95% CI 88.2–99.9).

3.3. Relationship between OS Biomarkers and the Severity of Brain Injury Assessed with MRI. The longitudinal multivariable model, adjusted for confounding factors, showed a significant independent association between AOPP levels (ln transformation) and MRI scores ($p = 0.006$, $B = 1.301$, CI 95% 0.38–2.22; Figure 4(a)), indicating that the increase of protein peroxidation levels during the first five days of life is associated with severe brain damage. In particular, males showed an increased risk of oxidative neurological damage, as comes to light from the significant correlation between AOPP and brain damage in males ($p = 0.005$, $r = 0.465$; Figure 4(b)). Using the ROC curve cut-off, a multivariable logistic regression model was performed, taking the MRI score as a dichotomic dependent variable. The last model confirmed that AOPP levels and male gender were both risk factors for more severe brain damage (AOPP: OR = 3.6, 95% CI 1.1–12.2 and gender: OR = 5.6, 95% CI 1.2–25.7, resp.). In detail, the increase of an AOPP logarithmic scale of 1 increases 3.6 times the risk of brain damage. As for the other two biomarkers, both regression models showed a negative association between the levels of NPBI (ln) and MRI score (linear model: $B = -0.94$, 95% CI -1.69 to -0.18; logistic model: OR = 0.20, 95% CI 0.06–0.71). No statistically significant association was found between the plasma levels of IsoPs and MRI score.

4. Discussion

Early objective diagnosis of brain injury is important for prognostication and decision-making in term newborns with HIE. Our study illustrates that infants who have suffered from severe birth asphyxia show increased OS levels compared with those who have had mild or moderate asphyxia. In particular, the increase of OS levels in the perinatal period was highlighted by a higher accumulation of plasmatic levels of AOPP and the index of membrane protein oxidative damage. The high levels of AOPP were significantly associated with an increase in brain damage, quantified with MRI. In our study, males, despite lower levels of OS at birth, were at the greatest risk of developing brain injury, showing an increased susceptibility to oxidative stress damage. The newborn brain is particularly susceptible to oxidative damage, both for the reduced antioxidant capacity and for the high consumption of oxygen and the high concentration of lipids and chemically reactive species, such as free iron [26, 27]. Free radicals, which are continuously produced in the course of the common cellular metabolic processes, greatly increase, especially at the cerebral level, after a hypoxic-ischemic event. Asphyxia and acidosis result in fact in the release of free iron in plasma, and free iron itself is responsible for further free radical formation, such as isoprostanes and AOPP. The neonatal brain, being particularly rich in polyunsaturated fatty

Table 3: MRI scores and outcome reported by groups.

Group	MRI score	Outcome
Mild/Moderate HIE		
1	7	Survived
2	1	Survived
3	1	Survived
4	0	Survived
5	0	Survived
Severe HIE		
1	11	Died
2	11	Died
3	10	Died
4	7	Survived
5	6	Died
6	5	Died
7	5	Died
8	5	Died
9	5	Died
10	5	Died
11	4	Survived
12	4	Survived
13	3	Survived
14	3	Survived
15	1	Survived
16	1	Survived
17	1	Survived
18	1	Survived
19	1	Survived
20	1	Survived
21	1	Survived
22	1	Survived
23	1	Survived
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41	0	Survived
42	0	Survived
43	0	Survived
44	0	Survived
45	0	Survived
46	0	Survived
47	0	Survived
48	0	Survived
49	0	Survived
50	0	Survived
51	0	Survived

acids, is particularly vulnerable to lipid peroxidation during asphyxia, making the immature myelin particularly susceptible to free radical attack [28]. The OS derived from the overproduction of free radicals dominates the lack of antioxidant mechanisms, including reduced activity of superoxide dismutase, catalase, and glutathione peroxidase (GPX), and it was thus involved in the pathogenesis of hypoxic-ischemic injury [29–31]. The increased production of GPX detected in the cerebrospinal fluid of neonates with HIE by Vasiljević et al. suggests that this is an active mechanism of response to oxidative stress induced by hypoxia-ischemia. Moreover, the same authors found that increased GPX activity correlates with the severity of the insult and hypoxic brain damage [31]. After HIE, the increase in OS occurs especially during the reperfusion and reoxygenation phase. In this phase, the free iron release and the activation of some prooxidant enzymes (such as the nitric oxide synthese, cyclooxygenase, lipooxygenase, and xanthine oxidase) determine a chain reaction, culminating in the production of free radicals, such as the highly toxic peroxynitrite, which cause cell damage through the peroxidation of membrane lipids and damage to DNA and proteins [32, 33]. The cascade activation of all these mechanisms leads to endothelial damage, with increased capillary permeability and therefore cytotoxic edema; soon after, the activation of caspases 3 and 9 causes the activation of apoptotic mechanisms [28, 29, 32, 34–36]. The higher the plasma AOPP, end products of protein peroxidation, and thus index of free radical damage, the more severe was the perinatal hypoxia-ischemia, confirming what has so far reported in the literature and emphasizing how proteins are the first target of toxic action of free radicals. An increase of carbonyl groups and expression of protein oxidation has been widely demonstrated and observed from the earliest 3 hours after the advent of hypoxia-ischemia [18, 37, 38]. The altered protein molecules act as a trap for free radicals, which further start chain reactions, worsening the damage. Often, this type of reactions is catalyzed by transition metals such as iron and copper, which enter the Fenton reaction [39]. Iron, in particular, is released from ferritin, although it may also derive from transferrin, hemoglobin, myoglobin, lactoferrin, and cytochromes. During hypoxia and subsequent reoxygenation, iron is moved from storage

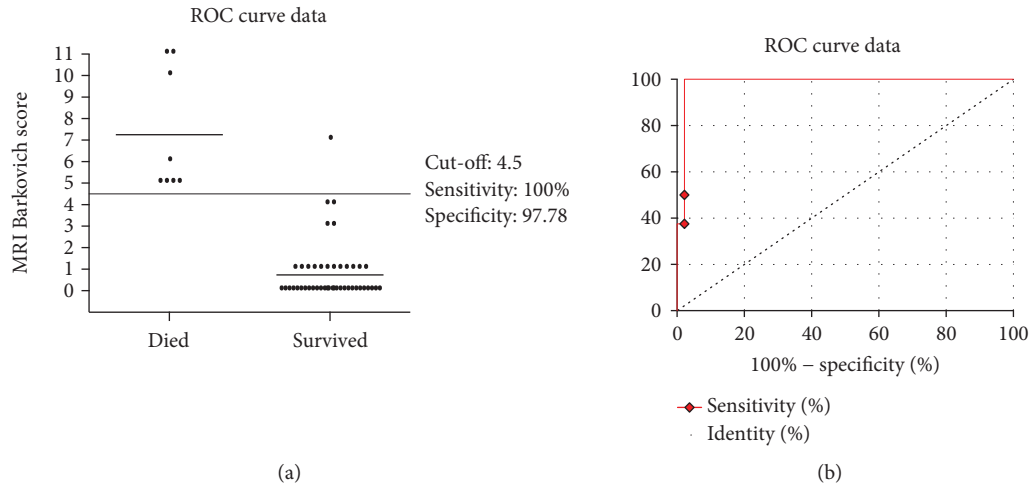

Figure 3: ROC curve analysis for the MRI score. The MRI score plotted curve indicated 4.5 as the best predictive threshold with a sensitivity of 100% (95% CI 63.06–100) and a specificity of 97.7% (95% CI 88.2–99.9). ROC curve discriminate newborns with a major risk to die from newborns surviving without impairments.

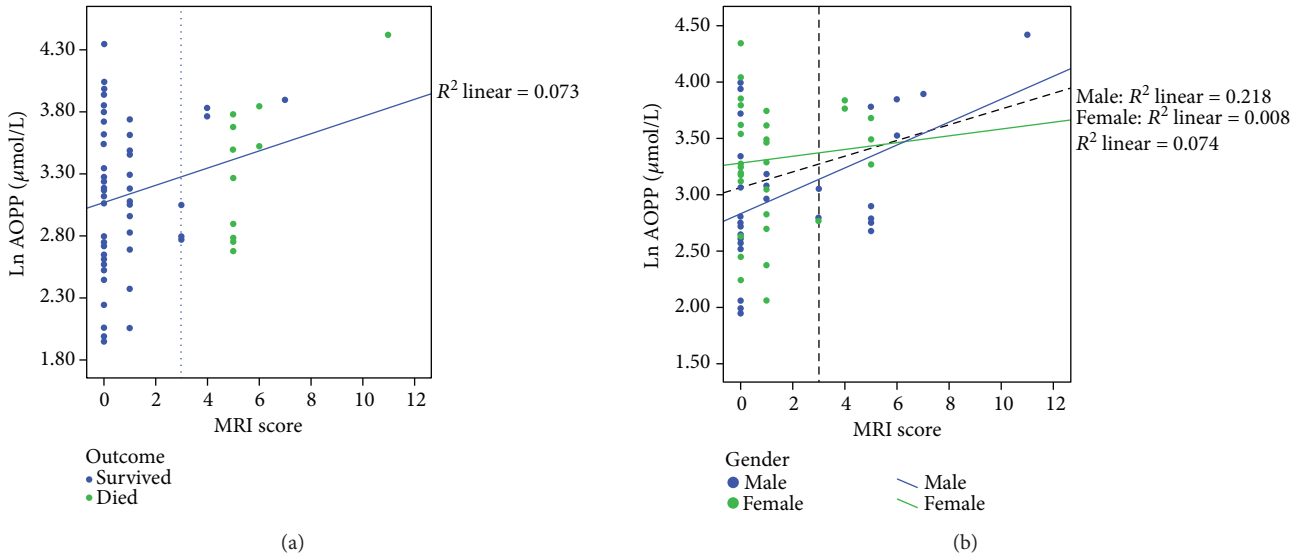

Figure 4: Scatter dot plots illustrate the relationship between the AOPP level (ln scale) and MRI score, reported, respectively, by short-term outcome (a) and by gender (b).

sites and converted to a form detectable in the plasma and extracellular space, so called "free iron" or NPBI. The increase in NPBI levels exposes proteins and membrane lipids to the attack of free radicals and thus to oxidative damage [37, 40]. In the literature, several studies have reported the role of free iron in the post anoxic oxidative damage. Ciccoli et al. have shown that erythrocytes of hypoxic infants released more iron than those of normoxic adults [41]. Buonocore et al. reported that the plasma level of NPBI is highly predictive of intrauterine suffering and brain damage [42]. In our study, NPBI levels were significantly higher in infants with severe perinatal asphyxia in the first six hours of life, while free iron plasma levels in this group of infants showed a downward trend, probably linked to the effect of hypothermia. Numerous studies in fact have shown that therapeutic hypothermia is effective not only in reducing brain damage and improving long-term outcomes but also in reducing levels of reactive oxygen species responsible for oxidative damage [38, 43]. As overproduction of free iron is known to be a consequence of hypoxia-induced acidosis, we hypothesized that the recovery of cellular metabolism during hypothermia will lead to a reduction of triggers of free iron release; meanwhile, AOPP and isoprostanes and expression of oxidative cellular damage persist to be increased in plasma.

Despite the significant difference highlighted in the levels of AOPP between boys and girls at birth, our study confirms what is already well known that there is a higher male susceptibility to brain oxidative damage. A recent European analysis on 4500 children with cerebral palsy revealed in fact that incidence of cerebral palsy is 30 times higher in males than in

females [44]. These gender differences in the immature brain seem to be related to intrinsic chromosomal and hormonal differences. The protective role of estrogen against OS and thus the fact that most males are susceptible to oxidative damage have been reported by numerous studies [45–47]. Giordano et al. demonstrated in an animal model that estrogens modulate the cerebral expression of some antioxidant enzymes (paraoxonase 1 and 2 (PON1 and PON2)), increasing in this way the resistance of female neurons to oxidative damage [46]. Furthermore, although the neuroprotective effect of estrogen is well known, the absence of the protective effect of estradiol in some cells of PON2-knockout mice ($PON2^{-/-}$) suggests that other mechanisms of neuroprotection related to female hormones may come into action against free radical damage [45, 48]. The presence of differences in the OS levels between males and females and their relationships with the hormonal status have also been emphasized by another study by Minghetti et al, in which males have a higher lipid peroxidation and reduced antioxidant capacity than females, contributing to the concept of male disadvantage in respect of the damage caused by free radicals [47]. Despite the advent of new studies and research projects, the exact mechanisms producing these gender differences are still largely unknown.

The role of oxidative stress in newborn morbidity with respect to the increased risk of free radical damage in these babies is growing. However, challenges remain in the early identification of infants at risk for neonatal encephalopathy, determination of timing and extent of hypoxic-ischemic brain injury, and optimal management and treatment [49]. Despite ongoing limits such as the need of a specific kit and specific instruments to measure oxidative stress biomarkers consequently associated with the need of an expert laboratory team and the high cost, making the process of reaching the goal slower, researchers are currently working to develop a biomarker panel, which can become useful to the clinicians as a point of care.

Literature on brain imaging of asphyxiated newborns typically uses MRI standard imaging techniques, performed following hypothermia at the end of the first week of life, to define the presence and extent of brain injury in these newborns and to provide a prognosis for subsequent neurological impairment [50]. DWI allows recognition of structural brain abnormalities during the first week, which only become clearly visible on conventional imaging by days 7–10 of life [51, 52].

The presence of an association between biomarkers of oxidative stress, measured in the first hours of life, and brain damage successfully evaluated through neuroimaging emphasizes the possibility of early identification of newborns at greater risk of brain damage and also underlines the validity of the AOPP, as products of OS damage in the plasma and therefore as biomarkers of neuronal damage. Knowing also that, after a hypoxic-ischemic insult, cellular damage on energy substrates continues to evolve over the first 12–48 hours, we suggest that the introduction of new neuroprotective strategies and antioxidants in such an early stage of life could change the long-term outcome of these infants.

Abbreviations

ADC: Apparent diffusion coefficient
aEEG: Amplitude integrated electroencephalography
AOPP: Advanced oxidation protein products
ASL: Arterial spin labeling
DWI: Diffusion-weighted imaging
F2-IsoPs: F2-isoprostanes
GPX: Glutathione peroxidase
HIE: Hypoxic-ischemic encephalopathy
^{1}H-MRS: Hydrogen magnetic resonance spectroscopy
HPLC: High-performance liquid chromatography
LC-MS/MS: Liquid chromatography tandem mass spectrometry
NIRS: Near-infrared spectroscopy
NPBI: Non-protein-bound iron
OS: Oxidative stress
PON1: Paraoxonase 1
PON2: Paraoxonase 2.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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Research Article

Urinary ^{1}H-NMR Metabolomics in the First Week of Life Can Anticipate BPD Diagnosis

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Despite the advancements in medical knowledge and technology, the etiopathogenesis of bronchopulmonary dysplasia (BPD) is not yet fully understood although oxidative stress seems to play a role, leading to a very demanding management of these patients by the neonatologist. In this context, metabolomics can be useful in understanding, diagnosing, and treating this illness since it is one of the newest omics science that analyzes the metabolome of an individual through the investigation of biological fluids such as urine and blood. In this study, 18 patients admitted to the Neonatal Intensive Care Unit of the Cagliari University Hospital were enrolled. Among them, 11 patients represented the control group and 7 patients subsequently developed BPD. A sample of urine was collected from each patient at 7 days of life and analyzed through ^{1}H-NMR coupled with multivariate statistical analysis. The discriminant metabolites between the 2 groups noted were alanine, betaine, trimethylamine-N-oxide, lactate, and glycine. Utilizing metabolomics, it was possible to detect the urinary metabolomics fingerprint of neonates in the first week of life who subsequently developed BPD. Future studies are needed to confirm these promising results suggesting a possible role of microbiota and oxidative stress, and to apply this technology in clinical practice.

1. Introduction

Neonates, and especially those born prematurely, are particularly vulnerable to oxidative stress, as their levels of antioxidant enzymes are inadequate and unable to protect the rapidly growing tissues, including the developing lung, from oxidative injury. Bronchopulmonary dysplasia (BPD) is one of the main complications of prematurity included in the spectrum of "oxygen radical diseases of neonatology" [1]. Oxidative stress plays a key role in the pathogenesis of respiratory diseases of the preterm newborns, as a consequence of inadequate antioxidant capacities and increased presence of multiple oxidative stimuli, which is caused by preterm birth, intensive care therapy, infections, and inflammatory status [2].

BPD is the most common pulmonary complication in preterm newborns and it represents one of the principal causes of death in the extremely preterm neonates born before the 32nd week of gestation or weighing less than 1500 g at birth. BPD is a very complex pathology and it has been extensively reviewed in terms of physiopathology, diagnosis, treatment, and prevention in recent years [3].

In the last decade, the progress of perinatal medicine allowed a better definition of the etiopathogenesis and the features of BPD, improving the survival rate of this class of preterm newborns, although to date it is still extremely difficult to resolutely and effectively treat newborns suffering from this illness [4].

Thus, the reduction of BPD incidence represents a fundamental step to reduce the harmful consequences of this

pathology and further reduce the incidence of other diseases that disrupt the pulmonary function and the neural development of the patients.

Therefore, being able to identify precociously the subjects at higher risk of BPD would allow for an improvement of the outcome of these little patients and would pave the way for individualized medicine [5].

Personalized medicine uses new medical technologies discovered in the last decade such as the "omics" sciences. Among them there is metabolomics, a multidisciplinary science that integrates several aspects derived from different branches (physics, chemistry, biology, and medicine).

Metabolomics is defined as "the quantitative measure of the multiparametric dynamic metabolic response of the living systems to physiopathological stimuli or genetics modifications" [6]. Thus, it is the science that studies the metabolome, namely, the complex system of metabolites that are the final products of the biochemical reactions, released in biological tissues and fluids. Studying the metabolites of an individual makes it possible to outline its biochemical phenotype [7–9], and in this sense, metabolomics is able to provide a high-resolution picture of what is happening inside an organism in a certain moment of its life. This provides a systematic study of the biochemical fingerprints resulting from cellular processes.

Moreover, it is also possible to accurately define a pathological state and perform a monitoring of the metabolic response of an organism to particular therapies, but one of the most interesting aspects is that it allows for the precocious identification of certain diseases even in the preclinical stage. A field where this appears extremely relevant is the neonatal period. In fact, metabolic profiling may provide information about the risk, prediction, diagnosis, and prognosis in a variety of diseases [10–13]. Therefore, metabolomics appears a relevant tool to provide useful information for the early identification and the management of complex pediatric disorders such as BPD. The search for a urinary metabolic profile distinguishing newborns who will develop BPD from preterm controls would allow the best management of the illness and its complications. We have already published a study analyzing urine at birth, in the development of BPD [14]. These data were confirmed by a study on the amniotic fluid [15]. Only in the present study, which focuses on urine samples collected at day seven of postnatal life, the potential influences after birth were investigated in the first week of life. The main objective of this study was to characterize the urinary metabolome of newborns affected by BPD using ^{1}H-NMR spectroscopy in order to detect a metabolic fingerprint to be used as a predictive and diagnostic tool for this pathology. In this respect, attempts have been made to find a unique metabolic profile that would discriminate preterms developing BPD from those who do not, in order to identify as early as possible who among neonates will later develop the pathology. It is known that oxidative stress is important in the pathogenesis of various types of neonatal diseases (e.g., BPD, respiratory distress syndrome, retinopathy of prematurity, necrotizing enterocolitis, and congenital malformation). However, there is a need for more information regarding the prediction and diagnosis of oxidative stress-related conditions in neonates. There is a lack of knowledge on the individual evolution when conditions of susceptibility to oxidative stress damage are present as in the case of preterm infants predisposed to BPD.

2. Patients and Methods

2.1. Patients. This study included 18 preterm newborns admitted to the Neonatal Intensive Care Unit (NICU) of Cagliari University Hospital from 1st January 2012 to 30th September 2014. The gestational age was under 28 weeks and the birth weight under 1500 g. Parents were informed of the purpose of the study and their written consent was obtained. The patients were subsequently divided into 2 groups: the first group (12 controls) comprised newborns not affected by BPD and the second group (7 patients) consisted of newborns that developed BPD. The first case group (3 males, 9 females) had a mean gestational age of 27.9 ± 0.9 weeks and a mean birth weight of 1017 ± 200 g. The study group (4 males, 3 females) had a mean gestational age of 27 weeks and a mean birth weight of 784 ± 87 g (see Supplementary Material (available here) for maternal and neonatal demographic information). A sample of urine was collected from each newborn (approximately 1 mL), using a noninvasive method at the seventh day of life. As previously described [11], a cotton ball was inserted into the disposable diaper; then urine was aspirated through the use of a syringe and transferred into a sterile 2 mL vial. After collection, all the vials were immediately frozen after the addition of sodium azide 0.1% (*w*/*v*) and stored at −80°C until metabolomics analysis.

2.2. Reagents. All chemicals used in this study were of analytical grade. Deuterium oxide (purity 99.9% in D) and the internal standard trimethylsilylpropanoic acid (TSP) for NMR analysis were purchased from Sigma-Aldrich (Milano, Italy). Deuterated phosphate buffer (pH 7.4; 1.5 M) with 10% TSP was prepared and used for pH adjustment.

2.3. NMR Analysis. A total of 540 mL of the urine samples was mixed with 60 mL of aqueous phosphate buffer solution 1.5 M (pH 7.4). TSP in D_2O was added to provide an internal reference for the chemical shifts (0 ppm). Samples were randomly analyzed as previously described [11]. Shortly, all ^{1}H-NMR spectra were acquired on a 500 MHz Inova (Varian Inc., Palo Alto, CA) spectrometer equipped with a 5 mm triple resonance probe with *z*-axis pulsed field gradients. One-dimensional ^{1}H-NMR spectra were collected at 27°C with a Presat 1D Noesy pulse sequence for water suppression. The relevant parameters used were calibrated and used as follows: a spectral width of 7997.6 Hz, acquisition time of 1.5 s, relaxation delay of 2 ms, 90° pulse of 10.7 ms, and number of scans of 128. The spectra processing was performed using MestReNova software (Version 7, Mestrelab Research S.L., Santiago de Compostela, Spain). The water resonance region, between 4.36 and 5.40 ppm, was excluded in order to eliminate the residual water signal, as well as that of urea, between 5.40 and 6.16. Subsequently, each spectrum was segmented into consecutive "buckets" of fixed size, 0.04 ppm, excluding the

TSP resonance region, and the corresponding spectral areas were integrated. The final area included the region between 0.4 and 10 ppm to perform a matrix in which rows are samples, called observation, and columns are buckets, called variables. The generated matrix was normalized to the total sum of integrated areas fixed to 100 in order to minimize the effect of different concentrations of urine samples [16].

2.4. Statistical Analysis. The final dataset was imported into the SIMCA-P (version 14.0, Umetrics, Sweden) program and was Pareto scaled prior to analysis. Principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA), unsupervised and supervised methods, respectively, were applied to the NMR dataset. PCA is a projection method used to obtain a general overview on the state of samples, highlighting possible clusters, trends, or outliers.

OPLS-DA is a classification method that maximizes the covariance between the measured data of the X variable (peak intensities in NMR spectra) and the response of the Y variable (class assignment) within the groups. The generated R^2Y and Q^2 values described the predictive ability and the fitting reliability, respectively. The model was validated by a permutations test ($n = 200$). The permutation test was used to check the validity and the degree of over-fit for the model. The importance of the discriminating variables has been indicated as VIPs (variables of importance on the projection). Using the VIP list, the most important variables were translated and identified by means of the Chenomx NMR Suite software (version 7, Chenomx Inc., Edmonton, Canada) and the HMDB database [17].

3. Results

A representative spectrum of the ^{1}H-NMR urine analysis is shown in Figure 1 pointing out the most relevant metabolites. The ^{1}H-NMR spectroscopy coupled with multivariate statistical analysis was applied in order to identify possible metabolic profiles characteristic of preterm newborns who developed BPD compared to preterm newborns who did not develop BPD. The PCA of the dataset did not show the presence of any clusters (unpublished results). Based on Hotelling's T^2 test at 95% confidence and DModX test, no samples were identified as outliers. Therefore, OPLS-DA was applied in order to identify different possible metabolic profiles between the two classes.

The OPLS-DA model is reported in Figure 2(a) (black dots: controls, grey dots: BPD). The results obtained showed that the samples were clearly separated into 2 groups indicating that controls and BPD-prone newborns presented a markedly distinct metabolic profile. The parameters of the model were $R^2Y = 0.81$ and $Q^2 = 0.66$. By being both higher than 0.5, these parameters supported the goodness of the model. To validate the OPLS-DA model, a permutation test of the corresponding PLS-DA model was performed. The significance of the model was assessed through 200 applications in which all Q^2 values of permuted Y vectors were lower than the original one and the regression of the Q^2 line had an intercept below zero (Figure 2(b)). The results clearly indicated that the OPLS-DA model was statistically valid.

The metabolites that discriminated the two classes were identified by analyzing the S-plot and the VIP list (unpublished). Variables (metabolites) with a VIP score > 1 were considered and determined using the Chenomx NMR Suite 7.1. The main discriminant metabolites are identified and reported in Table 1. In detail, the metabolites contributing to the separation between controls and BPD were alanine and betaine (increased in the BPD group), trimethylamine-N-oxide (TMAO), and lactate and glycine (decreased in the BPD group).

4. Discussion

The main objective of our study was to characterize the urinary metabolome of newborns affected by BPD in order to identify a metabolomics fingerprint to be used as a predictive and diagnostic tool for this pathology. In our study population, the ^{1}H-NMR analysis of the urine samples demonstrated that the preterms who go on to develop BPD show a different metabolic profile compared to the healthy controls.

In particular, the multivariate analysis of the urine spectra highlighted a cluster distribution, with a significant separation of the samples of the 2 groups of preterm neonates. In a previous work by Fanos et al. in 2014, the urinary metabolic profile in the first hours of life of extremely preterms who subsequently developed BPD was studied. In this study, it was hypothesized that the cellular metabolic activity associated with this condition could generate a distinctive model of metabolites, distinguishable from the metabolic profile of healthy newborns. Furthermore, it was also suggested that the destruction of the airways characterizing BPD could be the cause of the extensive cellular stress [14]. Even though there are some differences in the resulting metabolites, the main result of the present study confirms the hypotheses of the previous one.

Alanine is a nonessential amino acid made in the body from the conversion of the carbohydrate pyruvate or the breakdown of DNA and the dipeptides carnosine and anserine, which functions as a major energy resource. In skeletal muscle, the synthesis of alanine is directly proportional to the intracellular concentration of pyruvate that mainly increases when there is a high rate of degradation of fatty acid for energetic purposes, with a subsequent slowdown of the TCA cycle and ketone body formation. Pyruvate, in anaerobic conditions, cannot be oxidized in the Krebs cycle, so it is converted both into alanine and lactic acid. These compounds are released in the circulatory system, thus the plasmatic increase of alanine could explain its increase in urines. Furthermore, the glucose-alanine cycle is stimulated by the increase of plasmatic levels of glucocorticoids (cortisol), in response to stressful events of physic origin, as the BPD occurrence. Alanine levels parallel blood sugar levels. Alanine is an important participant and a regulator of glucose metabolism. It has been shown that intravenous glucose loading in infants with BPD resulted in a net increase in resting energy expenditure [18]. For this reason, the risk of pulmonary stress caused by an increase in basal oxygen

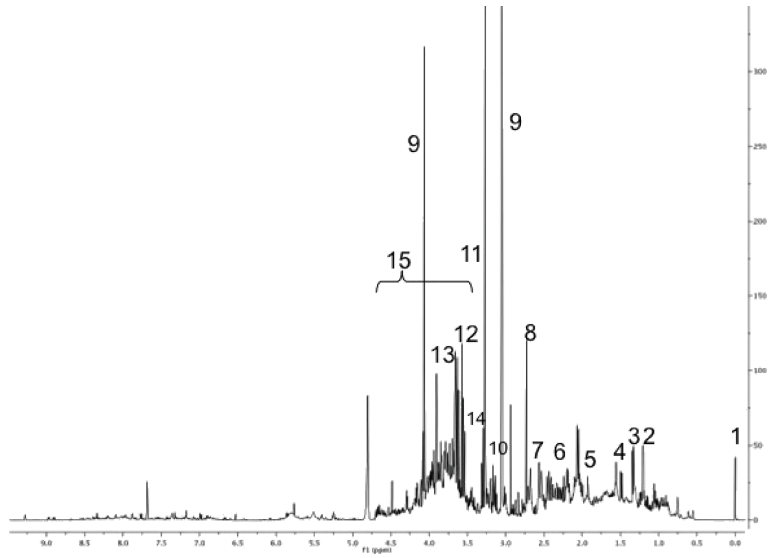

Figure 1: ^{1}H-NMR spectra of urine samples from a preterm infant with the following main assignments: 1 = TSP; 2 = 3-hydroxybutyrate; 3 = lactate; 4 = alanine; 5 = acetate; 6 = succinate; 7 = citrate; 8 = dimethylamine; 9 = creatinine; 10 = betaine; 11 = trimethylamine-N-oxide (TMAO); 12 = myoinositol; 13 = glycine; 14 = betaine; and 15 = glucose.

consumption and carbon dioxide production resulting from glucose load has been suggested and our observed level of alanine might be linked to a deregulation of glucose and oxygen metabolism.

Lactic acid plays a role in several biochemical processes. Lactate is the end product of anaerobic glucose metabolism. The decrease in lactate urinary concentration in BPD patients could also be related to alterations in the Krebs cycle and Cory cycle. The study of Fanos et al. pointed out that during the first 24 hours of life, in newborns that will develop BPD, there is an increase in lactate level due to the activation of anaerobic glycolysis in response to the reduction of oxygen levels [14]. After 7 days of life, as demonstrated by the present study, urinary levels of lactate were decreased compared to those of controls, probably due to a compensatory response. In fact, the lactate at this stage can be transported to the liver and used as a gluconeogenetic precursor (Cory cycle). A decrease in lactate can be associated with increased oxidative stress. As lactate can have a protective effect by decreasing the formation of reactive oxygen species or the production of superoxide anion, the decrease in our patients can suggest the presence of an oxidative stress condition [19].

In our study, the concentration of betaine was increased in BPD patients. Betaine is a product of choline oxidation and a donor of methyl groups, and it is involved in numerous biological processes, in particular in the DNA methylation.

Methylation and sulfhydryl groups play a pivotal role in different cellular functions such as DNA control, signal transductions, and metabolic pathways. Oxidative stress has a significant impact on these mechanisms [20, 21]. Methyl donors are necessary for the metabolic pathway that produces S-adenosyl-methionine, which is the universal donor of methyl groups, thus it is essential for the methylation process of the DNA [22]. In the study performed by Wang et al., it has been shown that the oral ingestion of D9-betaine is related to the generation of circulating D9-TMAO regardless of the intestinal flora [23]. TMA, the precursor of TMAO, is not produced via intermediary metabolism; rather, it depends on intestinal microbial breakdown of choline and other precursors. Thus, the intestinal microbiota has an obligatory role in the formation of TMAO [24, 25] and plays a pivotal role in TMAO production [26]. For this reason, the decreased levels of TMAO in the urines of BPD patients suggest an alteration of the intestinal microbiota. Since we found this metabolite increased in our previous study at birth, it is plausible that microbiota have modified qualitatively in order to influence maturation processes.

Concerning glycine, its urinary levels in neonates affected by BPD was lower compared to those in controls. Glycine plays a key role in glutathione synthesis, which is a powerful antioxidant of free radicals and other oxygen reactive species. Several pathologies of the preterm, such as BPD, are related to oxidative stress with consequent low levels of glutathione [27]. Furthermore, glycine intervenes in the processes of detoxification of the organism, since it can conjugate with several harmful substances such as benzoic acid, to form nontoxic compounds eliminable in the urine, such as hippuric acid [28]. An antioxidant role of glycine has been suggested. Glycine ameliorated oxidative stress and the impairment in antioxidant enzyme activities, inhibited NF-κB activation, and prevented expression of iNOS [29]. The observed decrease in glycine (and lactate) might indicate the presence of an oxidative stress condition or a lack of antioxidant defense.

High levels of hippuric acid were found in newborns suffering from recurrent respiratory tract infections (RRI), for which BPD is a predisposing factor. Bozzetto et al. in 2017 performed the metabolomics analysis of the urine samples of a children cohort suffering from RRI, showing that regarding the healthy controls there is an increase of hippuric acid. The latest is related to the specific composition of intestinal microbiota that seems to be altered in RRI children [30].

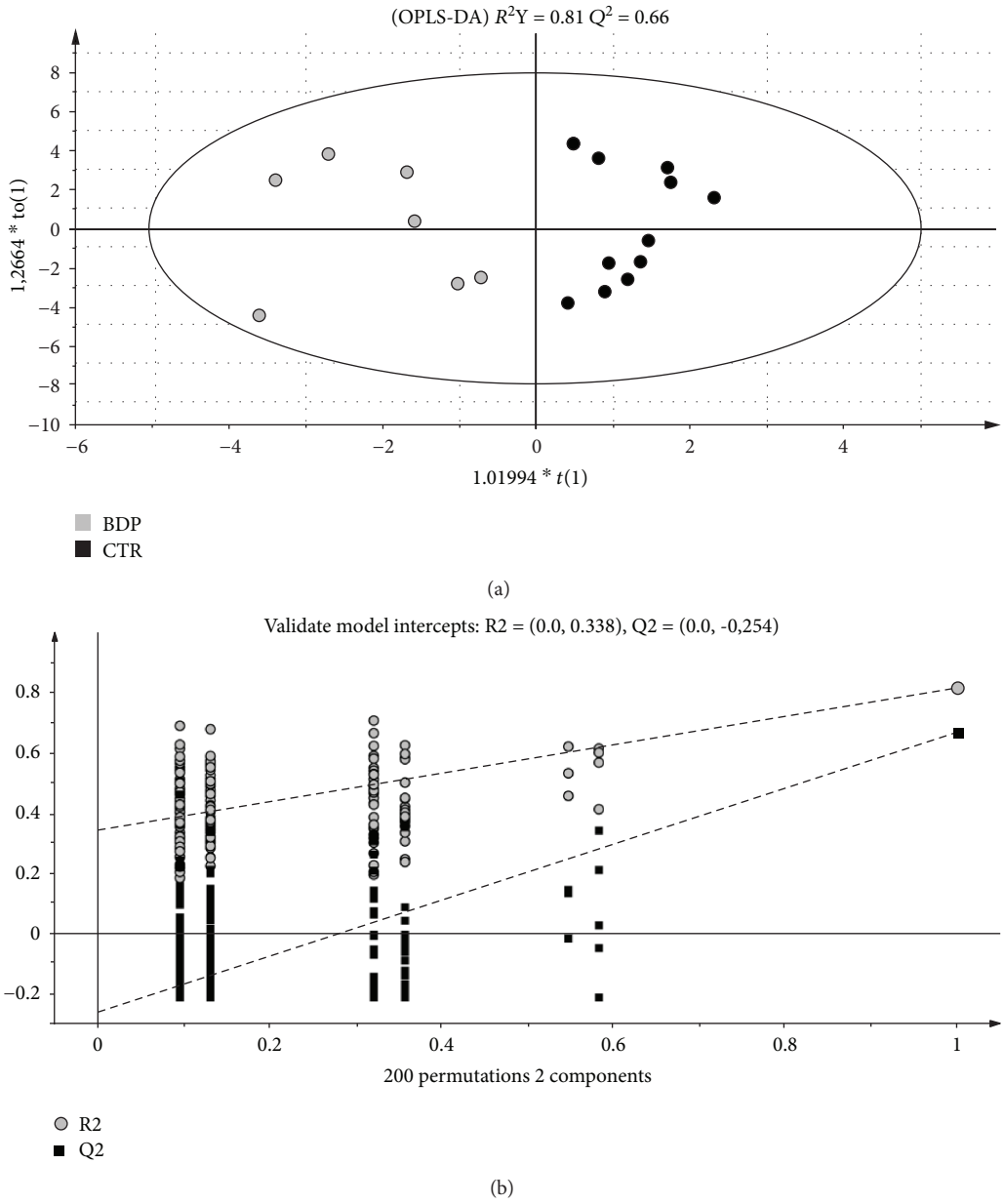

Figure 2: (a) OPLS-DA score plot of preterm controls (black circles) versus preterms who will develop BPD (grey circles) urine samples collected one week after birth. (b) The validation test of the model was assessed through 200 applications.

Table 1: Discriminant metabolites in urine collected one week after birth, between preterm controls and preterms who will develop BPD.

Metabolites	Chemical shift (ppm)	Trend in the BDP group
Alanine	1.48	↑
Betaine	3.25	↑
TMAO	3.27	↓
Lactate	1.34	↓
Glycine	3.57	↓

TMAO = trimethylamine-N-oxide.

In addition to this, the microbiota of the airways is capable of modifying the normal development of the pulmonary immune system, giving rise to dysbiosis of the microbiota itself that predisposes to the occurrence of pulmonary pathologies such as BPD [31]. Differences in airway microbiota composition are also related to the different metabolic profiles of the exhaled breath condensate (EBC). It has been previously observed through metabolomic analysis that adolescents affected by BPD maintain in the EBC a metabolic profile different from the healthy controls [32].

The results of our study support all these hypotheses since the low urinary levels of glycine found in BPD newborns could be correlated with the necessity to not eliminate this amino acid through urine since it is fundamental in

different biological processes such as those of detoxification and maintenance of microbiota homeostasis.

When we published our first study, we were concerned about the possibility to predict the outcome and the development of BPD at 6 weeks of life by an examination of urine samples at birth. In fact, the title contained a question mark. Our data suggested a genetic component in the determinism of BPD (already well-known in literature), associated to an intrauterine epigenetic component such as oxidative stress and inflammation [4]. The finding of Baraldi et al. in the amniotic fluid seems to support our hypothesis [15]. This study confirmed the involvement of some urinary metabolites already previously found (TMAO, lactate) indicating a possible role of microbiota that is realistically modified in the first week of life.

5. Conclusions

Our results identified a urinary metabolic fingerprint of the newborns suffering from BPD in the first week of life. The ^{1}H-NMR metabolomics analysis of preterm newborns' urine established a trend of the metabolites in patients that will develop BPD when compared to healthy controls.

The results of this preliminary study, could pave the way to a potential prediction and early diagnosis of BPD.

Future studies should investigate the diagnostic value of a metabolomics approach to define the evolution of the preterm infants in relation to BPD and other oxidative stress-associated disorders.

Data Availability

All data generated or analyzed during the study are included in this article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

Tables showing maternal and neonatal demographic information of the two groups of newborns. *(Supplementary Materials)*

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