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► **To cite this version:**

Carmen Peña-Bautista, Thierry Durand, Claire Vigor, Camille Oger, Jean-Marie Galano, et al.. Non-invasive assessment of oxidative stress in preterm infants. *Free Radical Biology and Medicine*, 2019, 142, pp.73-81. 10.1016/j.freeradbiomed.2019.02.019 . hal-02617362

HAL Id: hal-02617362

<https://hal.science/hal-02617362>

Submitted on 2 Jun 2021

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Non-invasive assessment of oxidative stress in preterm infants

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ARTICLE INFO

Keywords:

Biomarker

Oxidative stress

Prematurity

Urine

Saliva

Preterm infant

Saliva

Oxidative stress

Urine

Diagnostic techniques and procedures

ABSTRACT

Preterm newborns have an immature antioxidant defense system and are especially susceptible to oxidative stress. Resuscitation, mechanical ventilation, intermittent hypoxia and apneic episodes require frequently oxygen supplementation which leads to oxidative stress in preterm newborns. The consequences of oxidative damage are increased short and long-term morbidities, neurodevelopmental impairment and increased mortality.

Oxidative stress biomarkers are determined in blood samples from preterm children during their stay in neonatal intensive care units especially for research purposes. However, there is a tendency towards reducing invasive and painful techniques in the NICU (Neonatal Intensive Care Unit) and avoiding excessive blood extractions procedures.

In this paper, it has been described some studies that employed non-invasive samples to determine oxidative stress biomarkers from preterm infants in order to perform a close monitoring biomarker with a significant greater predictive value. Among these methods we describe a previously developed and validated high-performance liquid chromatography tandem mass spectrometry method that allow to accurately determine the most reliable biomarkers in biofluids, which are non-invasively and painlessly obtained.

1. Introduction

1.1. Oxidative stress in preterm infants

Oxidative stress is most commonly triggered by reactive oxygen species (ROS) (e.g. hydroxyl radicals, superoxide anions, hydrogen peroxides) generated under certain physiologic conditions in the mitochondria and other cell organelles (peroxisomes, Golgi, endoplasmic reticulum). ROS are originated as a consequence of a normal leak amounting 2% of the electrons transported along the electron transport chain (ETC), and in the endoplasmic reticulum or nuclear and cell membranes, but there are also generated when the antioxidant system renders incapable of scavenge ROS in excess [1–3]. In addition, enzymes, such as NADPH oxidases are great generators of ROS and constitute one of the most important sources of hydrogen peroxide [1,3]. Free radicals are highly reactive chemical species that instantaneously interact with nearby existing biomolecules in the cell (proteins, lipids, DNA and others) producing oxidized derivatives [4–6]. Some of them, show high reactivity and may form adducts with proteins, as well as oxidize other biomolecules, altering specific molecular pathways [7]

(see Fig. 1).

The main causes of oxidative stress in the newborn period are associated with oxygen administration [8], mechanical ventilation [9], intermittent hypoxia and apnea episodes [10], and asphyxia [11]. In general, the fetal-to-neonatal transition is characterized by intermittent hypoxia periods, involving over-production of oxidative substances and a disequilibrium in important cellular pathways, such as oxidation or inflammation biomolecules [12]. Preterm newborns have an immature antioxidant defense system and are, therefore, prone to oxidative damage. As a consequence, preterm infants will develop some conditions related to the production of free radicals with short-and-long-term consequences [13]. In this sense, recent research has focused on the impact of perinatal oxidative stress on the human brain development and its associated morbidities [14,15]. Moreover, these oxidative stress conditions are related to some chronic pathologies such as necrotizing enterocolitis (NEC) [16,17], bronchopulmonary dysplasia (BPD) [18–20], retinopathy of prematurity (ROP) [21], cardiac dysfunction [22], and acute kidney injury [23]. Therefore, oxidative stress monitoring and control would be highly desirable in the neonatal period to avoid its negative consequences and to improve outcomes of preterm

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List of abbreviations

AA	Amino acids	LO	Limited oxygen strategy
AGE	Advanced glycation end products	LP	Light protected
AOPP	Advanced oxidation protein products	MDA	Malondialdehyde
AscOOH	Ascorbylperoxide	NEC	Necrotizing enterocolitis
BAP	Biological antioxidant capacity	NeuroF	Neurofurans
BPD	Bronchopulmonary dysplasia	NeuroP	Neuroprostanes
2-dG	2'-deoxyguanosine	NICU	Neonatal Intensive Care Unit
EIA	Enzyme immunoassay	NO	Nitric oxide
ELISA	Enzyme-linked immunosorbent assay	NOx	Nitric oxide stable metabolites
ETC	Electron transport chain	NPBI	Non-protein bound iron
FM	Formula milk	3-NT	3-nitrotyrosine
FRAP	Ferric reducing antioxidant power	MVP	Multivitamins
GST	Glutathione S-transferase	8-OHdG	8-Oxo-2'-deoxyguanosine
GSH	Glutathione	O-Tyr	Ortho-tyrosine
HCA	Histological corioamnionitis	PF	Preterm formula
HELL	Ne-(hexanoyl) lysine	PGE2	Prostaglandin E2
HM	Human milk	Phe	Phenylalanine
HOX	High oxygen strategy	PrCarb	Protein carbonyls
HPLC	High performance liquid chromatography	ROI	Reactive oxygen intermediates
hsPDA	Significant hemodynamically patent ductus arteriosus	ROP	Retinopathy of prematurity
IL-6	Interleukin 6	ROS	Reactive oxygen species
IsoF	Isofurans	SOD	Superoxide dismutase
IsoP	Isoprostanes	TAC	Total antioxidant capacity
8-isoPGF	8-iso-prostaglandin F2 alpha	TBARS	Thiobarbituric acid reactive substances
IVH	Intraventricular hemorrhage	TH	Total hydroperoxides
LBW	Low birthweight	TXB2	Tromboxane B2
LE	Lipid emulsion	VLBW	Very low birth weight
		VU	Vascular underperfusion

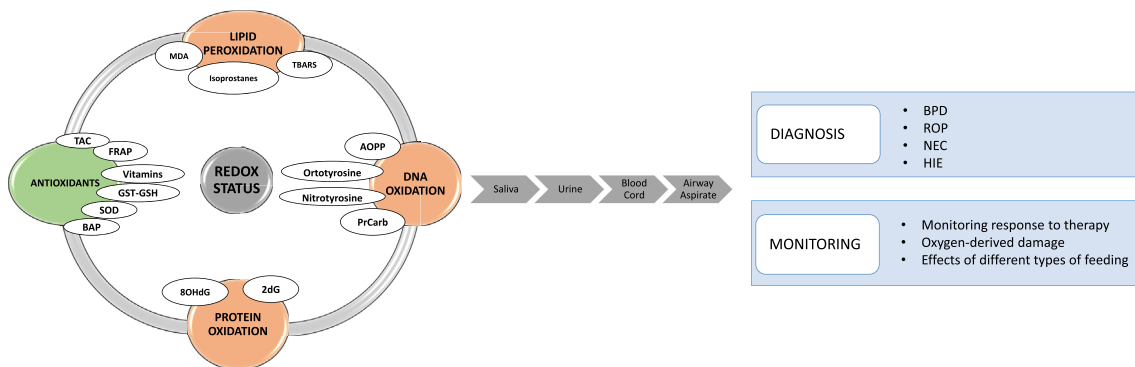


Fig. 1. Diagram representing the oxidative stress biomarkers determined in non-invasive biological samples.

children. In the last few years, some potential treatments that could reduce oxidative stress levels in preterm infants have been studied, such as melatonin or surfactant, but more clinical trials are required in order to confirm their clinical applicability [24].

1.2. Oxidative stress assessment and management

There is an increasing need in the development of reliable tools to evaluate, control, and reduce oxidative stress in preterm newborns [25]. A promising evaluation at molecular level is based on biomarkers obtained from biomolecules oxidation [26].

Biomarkers derived from lipids peroxidation play an important role in brain damage assessment, since brain is characterized by high lipid composition and oxygen consumption. Among these compounds, malondialdehyde (MDA) has been the most studied, and it has been determined in plasma and bronchoalveolar lavage samples from preterm newborns [27–29], using the thiobarbituric acid reactive substances (TBARS) assay. Thus, high levels of TBARS were found in studies

carried out with neonatal blood samples a few days after birth [30]. However, most of the TBARS formed *in vivo* are not related to lipid oxidation [31]. In addition, plasma and serum isoprostanes offer a reliable measurement of *in vivo* oxidative stress [32,33], and constitute biomarkers that are increasingly used. Regarding DNA and protein oxidation biomarkers, 8-hydroxy-2'-deoxyguanosine (8-OHdG) and carbonyl proteins have been the most studied compounds, respectively [34]. In this sense, 8-OHdG is used as oxidative stress biomarker from blood and tracheal aspirates samples [35,36]. Also, some protein oxidation products, such as carbonylated proteins, are evaluated as oxidative stress biomarkers from plasma and bronchoalveolar lavage fluid samples [37,38].

In general, most of the studies have evaluated oxidative stress in blood samples, and a few of them used tracheal aspirates and bronchoalveolar lavage fluid samples. However, the invasiveness and pain derived from these sampling procedures limit their sequential use for monitoring evolving conditions.

Therefore, non-invasively obtained samples could allow to monitor

Table 1
Non-invasive biomarkers to evaluate the oxidative stress levels in preterm infants.

Reference	Participants	Biomarker	Sample	Analytical technique	Results
[41]	LBW-1 + mother corticosterone treatment (n = 103) LBW-1 (n = 22)	Stimulated granulocyte ROI GSH IL-6	Cord blood	Flow cytometry, spectrophotometry, enzyme-linked immunosorbent assay	IL-6: LBW-1 + mother with corticosterone treatment > LBW-1 (34.20 pg/ml > 30.93 pg/ml, p = 0.97). Complete corticosterone treatment < not complete treatment (30.16 pg/ml < 54.83 pg/ml, p = 0.024) * ROI: LBW-1 + mother with corticosterone treatment < LBW-1 (0.40 nmol/106 cel < 0.50 nmol/106 cel, p = 0.251) Complete corticosterone treatment < not complete treatment (0.40 nmol/106 cel < 0.50, p = 0.039 nmol/106 cel) * NPBI: Preterm with NEC > preterm. (2.21 (µmol/L) > 0.95 (µmol/L), p = 0.012) * AOPP: Preterm with NEC > preterm (28.05 (µmol/L) > 15.80 (µmol/L), p = 0.048) * TH: Preterm with NEC > preterm. (154.48 (UCARR/L) > 107.40 (UCARR/L), p = 0.035) * IsoPs: VU > preterm (p = 0.022)*, HCA > preterm (0.003)* NPBI: VU > (preterm, preterm with HCA) (7.34 (µmol/L) > 0.94 (µmol/L), p = 0.007)* AOPP: VU > (preterm, preterm with HCA) (3.64 (µmol/L) > 2.71 (µmol/L), p = 0.007)* TBARS: preterm with sepsis > preterm (10.1 (nmol/mg protein) > 4.2 (nmol/mg protein), p < 0.01) * PrCarb: preterm with sepsis > preterm (2.4 (mmol/mg protein) > 1.1 (mmol/mg protein), p < 0.01) * PrCarb: Preterm < Control (0.048 (nmol/mg protein) < 0.105 (nmol/mg protein), p = 0.004) * MDA: Preterm > Control (2.47 (µM) > 1.83 (µM), p < 0.0001) * Selenium beneficial role against Cadmium oxidative stress induction is not only depending on concentration but also by its chemical structure. Correlation between gestational age and TAC (rho = 0.26, p = 0.0008)* and AGE (rho = 0.35; p < 0.0001)* and not for TBARS (rho = -0.10, p = 0.20) nor FRAP (rho = 0.05, p = 0.54) SOD: preterm < control (0.49 (IU) < 1.96 (IU), p < 0.001)* MDA: preterm < control (1.06 (IU) < 0.94 (IU), p = 0.75) Catalase: preterm < control (1.95 (IU) > 1.91 (IU), p = 0.90) TAC: preterm < control (3.69 (IU) < 3.85 (IU), p = 0.51) Lower oxidative stress levels were related to Chronic lung disease (p = 0.03) 8-OHdG: LBW (1,2,3) > Control (32.421 (pg/ml) > 4.15 (pg/ml), p < 0.005) * MDA: LBW (1,2,3) > Control (2.169 mM > 0.663 mM, p > 0.005)*
[42]	Preterm (n = 303) Preterm with NEC (n = 29)	NPBI AOPP TH	Cord blood	HPLC, spectrometry and spectrometry	15-F _{2t} -IsoP: males > females (36.2 (pg/ml) > 23.5 (pg/ml), p < 0.005)* TAC: males > females (1488 (µmol/L) > 1437 (µmol/L)) not significant differences. 8-OHdG: newborns born through meconium-stained amniotic fluid > newborns with clear liquor (55.36 (pg/ml) > 24.60 (pg/ml), p < 0.0001)* MDA: newborns born through meconium-stained amniotic fluid > newborns with clear liquor (2.43 (nmol/L) > 0.94 (nmol/L), p < 0.0001) * BAP/TH: LOX > HOX (13 (µM/UCARR) > 8 (µM/UCARR), p < 0.01)* PrCarb: Control < LBW (1,2,3) (2.409 (nmol/mg) < 3.559 (nmol/mg), p < 0.005)* MDA: Control < LBW (1,2,3) (0.728 (nmol/L) < 1.885 (nmol/L), p < 0.005)* Vitamin A: Control > LBW (1,2,3) (27.022 (mg/dl) > 20.954 (mg/dl), p < 0.005)* Vitamin E: Control > LBW (1,2,3) (0.699 (mg/dl) > 0.323 (mg/dl), p < 0.005)* Vitamin C: Control > LBW (1,2,3) (1.704 (mg/dl) > 0.743 (mg/dl), p < 0.005)* TAC: Control > LBW (1,2,3) (2.076 (mmol/L) > 1.336 (mmol/L)) *
[44]	Preterm (n = 31) Preterm with VU (n = 48) Preterm with HCA (n = 41)	IsoPs NPBI AOPP	Cord blood	LC-MS/MS HPLC-DAD Spectrophotometry	
[45]	Preterm with sepsis (n = 40) Preterm (n = 80)	TBARS PrCarb	Cord blood	Spectrophotometry	
[46]	Preterm (n = 54) Control (n = 43)	PrCarb MDA	Cord blood (plasma)	ELISA HPLC	
[47]	Newborns (n = 250)	MDA 8-OHdG	Cord blood Urine	HPLC ELISA	
[48]	Preterm (n = 165)	TAC FRAP TBARS AGE	Cord blood	Spectrophotometry	
[49]	Preterm (n = 25) Control (n = 25)	Catalase SOD TAC 8-OHdG	Cord blood Urine Cord blood (serum)	Spectrophotometry EIA ELISA	
[50]	Preterm (n = 31)	8-OHdG	Cord blood Urine	EIA	
[51]	LBW-1 (n = 14) LBW-2 (n = 22) LBW-3 (n = 19) Control (n = 24)	8-OHdG MDA	Cord blood Urine Cord blood (serum)	ELISA	
[52]	Preterm twins (n = 80)	15-F _{2t} -IsoP TAC	Cord blood (plasma)	EIA Spectrophotometry	
[53]	Newborns born through meconium-stained amniotic fluid (n = 59) Newborns born through clear liquor (n = 50)	8-OHdG MDA	Cord blood	ELISA Spectrophotometry	
[54]	Preterm + LOX (n = 44) Preterm + HOX (n = 44)	TH BAP	Cord blood	Spectrometry	
[55]	LBW-1 (n = 16) LBW-2 (n = 35) LBW-3 (n = 73) Control (n = 116)	MDA PrCarb TAC Vitamin A, E and C	Cord blood	Spectrophotometry	

(continued on next page)

Table 1 (continued)

Reference	Participants	Biomarker	Sample	Analytical technique	Results
[56]	Sick preterm (n = 17) Preterm (n = 7) Control (n = 8) LBW-1 with IVH (n = 15) LBW-1 (n = 110)	GST	Cord blood Blood 3h after birth Cord blood	Spectrophotometry	GST: (Sick preterm + Preterm) > Control (10.6 > 8.3 > 6.7 (U/g Hb), p = 0.03)
[57]	Preterm + BPD (n = 36) Preterm (n = 24) Preterm + HM (n = 29) Preterm + PF (n = 34) Control (n = 20)	ROI GSH	Urine	F/low cytometry Spectrophotometry	GSH: LBW-1 with < IVH LBW-1 (67.25 (mg%) < 71.03 (mg%), p = 0.337) Basal ROI: LBW-1 with < IVH LBW-1 (0.50 (nmol × 106 cells) < 0.40 (nmol × 106 cells), p = 0.045)* 8-OHdG on day 7: Preterm + BPD > Preterm (2.8 > 1.6, ng/mg Creatinine, p = 0.002)* 8-OHdG/2 dG ratio: preterm + PF > Preterm + HM (10.46 > 9.05, p < 0.02)* O-Tyr/Phe: preterm + PF > Preterm + HM (14.90 > 12.53, p < 0.01).*
[58]	Preterm (n = 30) Control (n = 30)	8-OHdG	Urine	ELISA	8-OHdG: Preterm > Control (p < 0.05) *
[59]	Preterm (n = 82) Control (n = 20)	8-OHdG/2 dG ratio O-Tyr/Phe ratio	Urine	HPIC-MS	Peroxide: Preterm > Control (10 μmol/mmol creatinine > 5 μmol/mmol Cr) * Peroxide: Preterm < Control (220.5 μmol/mmol creatinine < 244 μmol/mmol Cr) Not significant differences. At base lines 8–16 μmol/L and after 48 of parenteral nutrition 13–145 μmol/L (p < 0.05)
[60]	Preterm (n = 80) + Dextrose and amino acids (n = 20) + Dextrose, amino acids, and lipid emulsion (n = 20) + Dextrose, amino acids, lipid emulsion, and multivitamins (n = 20) + Dextrose, amino acids, lipid emulsion, MVP, and trace elements (n = 20) Each group was subdivided into photo-protected and photo-exposed subgroups (n = 10 each).	8-OHdG	Urine	ELISA	
[61]	Preterm (n = 65) LOX (n = 143) HOX (n = 120) Control (n = 32) LOX (n = 133) HOX (n = 120)	Peroxides Nitrate/nitrites	Urine	Fluorometric method Chemiluminescence	
[62]	Preterm (n = 80) + Dextrose and amino acids (n = 20) + Dextrose, amino acids, and lipid emulsion (n = 20) + Dextrose, amino acids, lipid emulsion, and multivitamins (n = 20) + Dextrose, amino acids, lipid emulsion, MVP, and trace elements (n = 20) Each group was subdivided into photo-protected and photo-exposed subgroups (n = 10 each).	Peroxides	Urine	Spectrophotometry	
[63]	Preterm (n = 65) LOX (n = 143) HOX (n = 120) Control (n = 32) LOX (n = 133) HOX (n = 120)	F ₂ -IsoPs IsoP Prostaglandins	Urine Urine	Mass spectrometry UPLC-MS/MS	F ₂ -IsoPs: Infants with 50% of their mother's milk fortified > Formula fed (p = 0.002)* Reference ranges for isoprostanes and prostaglandins were established for extremely low gestational age neonates.
[64]	Preterm (n = 65) LOX (n = 143) HOX (n = 120) Control (n = 32) LOX (n = 133) HOX (n = 120)	IsoP Isof NeuroP NeuroF	Urine	UPLC-MS/MS	IsoP, Isof, NeuroP, NeuroF neonatal ranges.
[65]	Preterm + AA (n = 16) Preterm + LE (n = 17) Preterm + LP (n = 19)	Vitamins B1, B2, B3, B6, C IsoP F _{2α} III	Urine	HPPLC LC-MS/MS LC-MS/MS	Vitamin B1: AA > LP > LE (1.48 > 1.21 > 1.01, (μg/μg creatinine) Vitamin B2: AA > LE = LP (0.03 μg/μg creatinine) Vitamin B3: AA < LE = LP (0.01 < 0.02 = 0.02, (μg/μg creatinine) Vitamin B6: LE > AA > LP (1.54 > 1.11 > 0.88, (μg/μg creatinine) Vitamin C: AA < LE < LP (2.04 < 2.86 < 3.04) p = 0.0005 for AA vs (LE, LP) * IsoP F _{2α} III: LE > AA > LP (283 > 241 > 183, nmol/mol creatinine) IsoPs: Before treatment > after pharmacological PDA closure (571 > 313, ng /mg creatinine., p = 0.006)
[66]	Preterm (n = 43)	IsoP	Urine	GC-MS	F ₂ -IsoP: decreased in Preterm + HM (p = 0.03) and increased in Preterm + PF (p = 0.01)*
[67]	Preterm + HM (n = 13) Preterm + PF (n = 9) Preterm with Cholestasis (n = 27) Preterm (n = 16) Preterm + ROP (n = 25) Preterm (n = 25) Preterm (n = 51) Preterm (n = 24) Preterm with mild IVH (n = 13) Preterm with severe IVH (n = 7)	IsoP TBARS 8-OHdG MDA AscOOH Allantoin	Urine Urine Urine Urine Urine Urine Urine	ELISA Spectrophotometry HPPLC LC-MS GC-MS	Cholestasis > not cholestasis (2591 < 3368, ng/mg creatinine, p = 0.93) 8-OHdG: preterm + ROP > Preterm (1.63 > 0.63 (/10 ⁶ dG), p < 0.001). * MDA: preterm + ROP > Preterm (1.97 > 1.38, (/10 ⁶ dG), p < 0.001) * AscOOH: Preterm with BDP > Preterm (0.037)* Allantoin levels increased since 36–48 h (0.098 μmol, p = 0.02) after birth in IVH while is stable in not IVH group.

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Table 1 (continued)

Reference	Participants	Biomarker	Sample	Analytical technique	Results
[81]	Preterm (n = 53) Control (n = 30)	8-isoPGF _{2α} NOX TXB2 PGE2	Urine	EIA Spectrophotometry EIA EIA	8-isoPGF _{2α} : Preterm < Control (10749.5 < 40476.4, pg/mL, p < 0.001) * NOX: Preterm that developed hspDA > nsPDA (221.0 > 165.5, μmol/L, p < 0.01) * Not differences between groups for TBX2 nor PGE2
[82]	Preterm (n = 16) Preterm + BPD (n = 9)	8-OHdG AOPP HEL	Urine	Enzyme-linked immunosorbent assay	8-OHdG at 5–8 days: (Preterm + BPD) > Preterm (18.8 > 11.9, ng/mg, p < 0.05)* AOPP: Preterm + BPD > Preterm. Not significant differences HEL: Preterm + BPD > Preterm. Not significant differences
[86]	Preterm (n = 16)	8-OHdG	Urine	LC	8-OHdG: oxygen supplementation > without oxygen supplementation (60.2 > 9.3, ng/mg creatinine, p < 0.05)*
[87]	Preterm (n = 50) Control (n = 16)	8-OHdG	Urine	ELISA	8-OHdG: Preterm > Control (27.4 > 18.2 μmol mol ⁻¹ creatinine, p = 0.0253)*
[88]	Control (n = 10) Healthy preterm (n = 21) Sick preterm (n = 16)	8-OHdG acrolein-lysine adduct nitrite/nitrate	Urine	ELISA ELISA Non-enzymatic assay	8-OHdG: sick preterm > health preterm > control (123.6 > 46.8 > 27.2, ng/mg Cr, p < 0.001) acrolein-lysine adduct: sick preterm > health preterm > control (689 > 372 > 241, nmol/mg creatinine, p < 0.05) Nitrite nitrate: sick preterm < health preterm < control (2.39 < 2.62 < 2.82, μmol/mg Cr)
[89]	Preterm + BM (n = 9) Preterm + FM (n = 18) Preterm + FM/BM mixtures (n = 6) Preterm + FM (n = 10) Preterm + FM (n = 10) LBW-1 (n = 35)	8-OHdG MDA	Urine Urine	HPLC-EC HPLC	8-OxodG: Preterm FM > Preterm FM/BM ((38.7 > 16.7 nmol/mmol creatinine, p < 0.0001)* Preterm FM > Preterm BM (38.7 > 11.6, nmol/mmol creatinine, p < 0.0001)* MDA: preterm + HM = Preterm + FM (0.074 = 0.078, μM/mM creatinine)
[91]		8-OHdG	Urine	ELISA	8-OHdG showed correlation with mental development index in very low-birthweight infants (coefficient = -0.44, p < 0.05)
[73]	Preterm with hypertensive mother (n = 32) Preterm with normotensive mother (n = 41)	8-isoPGF _{2α} MDA NO	Airway aspirates	Spectrophotometry	MDA: hypertensive mother < normotensive mother (1 < 3.7, μM, p = 0.097) NO: hypertensive mother < normotensive mother (9.1 < 12.2, μM, p = 0.496)

levels of biomarkers with desired frequency, without causing pain or distress to the infants and thus, providing clinicians with very relevant information [39]. In addition, the antioxidant profile evaluation would allow a personalized treatment avoiding oxidative stress related pathologies [40].

The aim of this article is to review oxidative stress biomarkers determined in non-invasive samples as oxidative status evaluation in preterm infants, which could be used as diagnosis, prognosis and monitorization of different treatments.

2. Non-invasive monitoring of oxidative stress in preterm newborns

In Table 1 we have summarized some studies that have determined oxidative stress biomarkers in non-invasively obtained samples from preterm infants.

2.1. Biomarkers in cord blood samples

Cord blood has been the most widely employed matrix in the evaluation of several mechanisms related to prematurity [41–57]. Among these studies, 8-OHdG, MDA, and protein carbonyls are the most used biomarkers, and they reflect DNA, lipid and protein oxidation, respectively [43,46,47,49–51,53,55]. Significant differences were observed for these biomarkers between preterm and term newborns showing higher levels of carbonylated proteins in preterm than in term infants (99.8 and 82.9 pmol mg⁻¹, respectively) [43]. In addition, different antioxidant compounds, such as vitamins, biological antioxidant potential or glutathione S transferase, have also been measured in cord blood samples, since their low levels favor the oxidative stress [48,52,54–57]. In fact, the ratio between biological antioxidant potential and total hydroperoxide (BAP/TH) showed correlation with the oxygen supplementation received by preterm newborns [54], and the preterm newborns showed correlation with gestational age at birth for total antioxidant capacity (TAC) and advanced glycation products (AGE) [48]. Despite the usefulness of cord blood samples to evaluate the oxidative stress during birth and its potential consequences in newborns, it is not useful to perform follow-up studies along some weeks/months after birth because new samples are needed in order to monitor changes along time. However, it could be useful in the prognosis of some pathologies such as NEC and sepsis [42,45].

2.2. Biomarkers in urine samples

Urine has rendered highly efficacious as non-invasively obtained biofluid. Some of the potential biomarkers in this matrix show usefulness in the prediction, monitoring or evaluation of the risk of suffering from different pathologies associated to oxidative stress. According to this, high peroxides and 8-OHdG levels were found in preterm infants [58–60], specifically higher peroxides levels were found in preterm with BPD than in preterm newborns without the pathology [58]. Also, levels of these biomarkers were higher in preterm that received parenteral nutrition, as well as in preterm compared to term infants [61,62]. Other biomarkers related to lipid peroxidation that have been determined in urine samples are isoprostanes [63–68], TBARS [69] and MDA [70]. Regarding isoprostanes, their determination is useful for ductus arteriosus ibuprofen treatment monitoring since their levels usually decrease after the treatment [67], as well as in the study of the beneficial effects of human milk since these newborns show lower levels than those fed with preterm formula [63,68]. In addition, the normality ranges of different isoprostanes families were established in randomized, controlled, and blinded clinical trials from preterm infants [65]. However, TBARS seem not to be useful in the diagnosis of cholestasis, showing similar values between preterm infants with and without the pathology [69]. Another study found that 8-OHdG, isoprostanes, and ascorbyl peroxide were useful biomarkers to evaluate

BPD risk in very low weight infants, a group susceptible to suffer this pathology [58,71]. In other studies, 8-OHdG and MDA were considered potential biomarkers of ROP [70], and allantoin was used to evaluate the risk for intraventricular hemorrhage in preterm newborns [72]. BPD was also monitored by means of the evaluation of MDA and NO levels in airway aspirates [73].

2.3. Biomarkers in saliva samples

Saliva is a promising matrix to evaluate oxidative stress in preterm newborns. Saliva sampling is easy, and it has shown applicability in different studies [74]. Hence, saliva has been used to measure melatonin levels in preterm newborns, being able to replace its determination in plasma samples [75].

3. Analytical methods development and applications

Most of the analytical methods applied to determine oxidative stress biomarkers in non-invasive samples are based on immunoassays, colorimetric assays, commercial kits and spectrophotometric measures (Table 1). These techniques show some disadvantages, such as, low sensitivity and specificity, high number of interferences and matrix effect, and low precision.

Recently, our group has validated reliable analytical methods to determine cortisol and isoprostanooids in non-invasive samples, such as saliva [76,77]. They are based on high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) and they are useful in the determination of 18 lipid peroxidation products, showing the advantages of high selectivity and sensitivity. These methods showed satisfactory limits of detection and quantification allowing the reliable determination of these compounds in saliva samples and reflecting oxidative stress damage. They have shown utility as potential biomarkers of neurodegenerative diseases, and currently they are being applied to the study of oxidative stress in ex-preterm infants, as well as to evaluate the lipid peroxidation in infants with autism spectrum disorders [77]. In addition, our group developed a new analytical method based on Surface-enhanced Raman spectroscopy to determine GSH in blood samples showing its capacity to obtain reproducible results in small sample volumes [78]. In fact, this method was applied to newborn cord blood samples showing results transferable to clinical practice for the oxidative stress study [79].

It is important to highlight that saliva samples have not been commonly used in the evaluation of oxidative stress damage in preterm newborns and this is a field that should continue growing. This easy sampling will allow the oxidative stress monitoring in this group of susceptible patients avoiding any additional pain.

3.1. Limitations of the analytical methods

Some of the developed methods that are used for research using non-invasive samples in preterm infants are based on mass spectrometry determinations [39,41,43,44,56,62,69]. However, these analytical methods are not suitable in the clinical practice since they are expensive and require specialized technician personnel. In this sense, it is important to develop more simple analytical methods to determine these biomarkers in non-invasive samples with suitable sensitivity and reproducibility in order to be clinically applied.

In addition, other studies used methods such as ELISA or spectrophotometry [45,46,48,50–52,55,57,66]. They are easily applicable to clinical practice but still need to be validated clinically, and their pathological values should be well established for the different diseases as potential biomarkers.

4. Oxidative stress monitoring proposal in the neonatal period

Non-invasive monitoring of oxidative stress damage in preterm

newborns could be reliably performed with salivary and urinary determinations of different oxidized products. Brain is one of the most important organs affected by oxidative stress in preterm newborns, and considering its high lipid composition, the determination of lipid peroxidation biomarkers in saliva or urine samples could be highly specific of neurological damage [80]. In this sense, it is important to highlight the determination of isoprostanes/neuroprostanes/dihomo-isoprostanes inasmuch as these compounds would reflect the *in vivo* oxidation of arachidonic, docosahexanoic and adrenic acid, respectively. Among these lipid peroxidation compounds, 8-isoPGF_{2α} levels in urine have been related to gestational age at birth [81], and, in general, they were considered useful markers of the oxidative stress status derived from feeding type during early life in premature infants [63,68]. A second important group of pathologies in these patients are those related to the respiratory system, such as BPD. For this, the determination of 8-OHdG in urine has shown good results being able to differentiate between healthy preterm and those with BPD [58]. In fact, Joung et al. found 8-OHdG levels of 2.8 and 1.6 ng mg⁻¹ creatinine, in healthy and BPD preterm infants, respectively, while Tokuriki obtained 18.8 and 11.9 ng mg⁻¹ creatinine in similar participants' groups. In both cases statistically significant differences were observed [58,82]. Nevertheless, isofurans are the main validated biomarkers for chronic lung diseases and BPD prediction as it was shown by Vento et al. [83], and Kuligowski et al. [65]. Also, airway aspirates could reflect respiratory system damage associated to BPD through the study of the microbiome in these samples, although this field would need further research to corroborate its utility and other biomarkers reflecting oxidative damage should be studied [84]. Until now, tracheal aspirates have been used and they could reflect oxidative damage caused by macrophages as an unspecific response to oxygen, inflammation and infection. In this regard, biomarkers such as 3-chloro-tyrosine and glutathione sulfonamide have been successfully employed since they show elevated levels in newborns who suffered from chronic disease (88 μmol mol⁻¹ tyrosine) compared to healthy newborns (49 μmol mol⁻¹ tyrosine) [85]. For ocular problems in preterm newborns such as ROP, analysis of tears could constitute a promising matrix for oxidative stress evaluation; however, no published experience for this purpose is available. Monitoring of processes such as ibuprofen treatment of patent ductus arteriosus has been carried out using serial isoprostanes determinations [67].

We conclude that for monitoring oxidative stress and associated damage in preterm newborns, urine could be a useful matrix in which lipid peroxidation, oxidized proteins and nucleic acid biomarkers could be easily and sequentially determined. Also, saliva and airway aspirates seem to be promising matrices for oxidative stress biomarkers determination that need to be validated before applying to research studies and clinical practice.

Conflicts of interest

The authors report no conflict of interest.

Acknowledgements

CC-P acknowledges a post-doctoral "Miguel Servet I" Grant (CP16/00082) from the Health Research Institute Carlos III (Spanish Ministry of Economy and Competitiveness).

CP-B acknowledges a pre-doctoral Grant (associated to "Miguel Servet" project CP16/00082) from the Health Research Institute Carlos III (Spanish Ministry of Economy, Industry and Competitiveness).

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