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"The follow-up of the critical infant and benchmarking: the improvement of morbidity through the critical analysis of perinatal data"

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LIST OF ABBREVIATIONS

AA	Arachidonic Acid
ADHD	Attention Deficit and Hyperactivity Disorder
AGEs	Advanced Glycated Endproducts
AKI	Acute Kidney Injury
ALB	Albumin
AOPP	Advanced Oxidation Protein Products
ASD	Autism Spectrum Disorder
BPD	BronchoPulmonary Dysplasia
CAT	Catalase
CI	Confidence Interval
CLD	Chronic Lung Disease
COX	Cyclooxygenase
CP (or ICP)	Cerebral Palsy (or Infantile Cerebral Palsy)
CP	Ceruloplasmin
CRP	C-Reactive Protein
CS	Cesarean section
CSF	Cerebrospinal fluid
CVI	Cerebral Visual Impairment
DCD	Developmental Coordination Disorders
DHA	Docosahexaenoic Acid
DOHaD	Developmental Origins of Health and Disease
ELBW	Extremely Low birth weight, < 1000 g
EOS	Early-onset sepsis
EPO	Erithropoietin
EPT	Extremely preterm
EUGR	Extrauterine Growth Restriction
FER	ferritin
FGR	Fetal Growth Restriction
FRD	Free-Radicals Disease
FRs	Free radicals
GA	Gestational Age
GBM	Gradient boosting machine
GC/MS	Gas chromatography–mass spectrometry
GDM	Gestational Diabetes Mellitus
GM	Grey matter
GMs	General Movements
GPX	Glutathione Peroxidase
GSH	glutathione reduced form
GSSG	glutatione oxidized form
1H-NMR	Proton nuclear magnetic resonance (proton NMR, hydrogen-1 NMR)
H_2O_2	Hydrogen Peroxide
HIE	Hypoxic-Ischemic Encephalopathy
HMDB	Human Metabolome Database
hsPDA	hemodynamically significant Patent Ductus Arteriosus
IQ	Intelligence Quotient
IS	Indoxyl sulfate
IsoFs	Isofurans
IsoPs	Isoprostanes
IUGR	Intrauterine Growth Restriction
IVH	Intraventricular Hemorrhage
LC-MS	Liquid Chromatography Mass Spectrometry
LGA	Large for gestational age
LOOH	Lipid hydroperoxides
LOS	Late-Onset sepsis
LPT	Late preterm (GA 34-36^{+6} weeks)
LTF	Lactoferrin
MB	Myoglobin
MBD	Metabolic Bone Disease
MBPs	Metal-Binding Proteins

MDA	Malondialdehyde
MEL	melatonin (N-acetil-5-metossitriptamina)
MLPT	Moderate-Late Preterm (GA 32-36^{+6} weeks)
MND	Minor Neurological Dysfunction
MPO	Myeloperoxidase
MPT	Moderate Preterm (GA 32-33^{+6} weeks)
MRI	Magnetic Resonance Imaging
MS	Mass Spectrometry
MT	Metallothioneins
NAC	N-acetylcisteine
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NEC	Necrotizing Enterocolitis
NFs	Neurofurans
NICU	Neonatal Intensive Care Unit
NPs	Neuroprostanes
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxid
NOS	Nitric Oxid Synthasis
NOX	NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase)
NPBI	Non protein-bound Iron
•OH	hydroxyl radical
O2−	superoxide anion radical
ONOO−	Peroxynitrite
OS	Oxidative Stress
OSI	Oxidative Stress Index
PCA	Principal Component Analysis
PCT	Procalcitonin
PDA	Patent Ductus Arteriosus
PE	Pre-eclampsia
Pg	Prostaglandin
PGF	Postnatal Growth Failure
PHH	Post-Hemorrhagic Hydrocephalus
PHVD	post-hemorrhagic ventricular dilatation
PLS-DA	Partial-Least Squares-Discriminate Analysis
ppm	parts per milion
pPROM	Preterm premature rupture of membranes
PUFAs	Polyunsaturated fatty acids
PVL	Periventricular leukomalacia
RDS	Respiratory Distress Syndrome
RF	Random Forest
rhSOD	Recombinant human superoxide dismutase
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SGA	Small for Gestational Age
SOD	Superoxide Dismutase
SVD	Spontaneous vaginal delivery
SVM	Support Vector Machine
TAC	Total Antioxidant Capacity
TCA	Tricarboxylic acid cycle
TF	transferrin
TH	Total Hydroperoxides
TMAO	Trimethylamine N-oxide
TOS	Total Oxidative Satus
VEGF	Vascular Endothelial Growth Factor
VLBW	Very Low Birth Weigh, < 1500 g
VPT	Very preterm
WISC	Wechsler Intelligence Scale for Children
WM	White matter
WPPSI	Wechsler Preschool and Primary Scale of Intelligence
XO	xanthine-oxidase
7,8-OHdG	7,8-hydroxy-2'-deoxyguanosine

CHAPTER 1

INTRODUCTION

1.1 Scientific evolution in neonatology: historical notes and epidemiological data

Neonatology represents a relatively recent branch of medicine and pediatrics, as it was only in 1960 that the same term was coined by Alexander Schaffer and the interest in the newborn dates back to a few decades earlier, as regards the transition to extrauterine life and special needs and pathological conditions typical of the neonatal age. The idea that a "premature" newborn, understood as immature or weak, could be cured and treated dates back to the second half of the 19th century, and since then some milestones of modern neonatology have led to a tumultuous variation in the epidemiological data of neonatal mortality and morbidity (Philip AG. 2005): the manteinance of thermal homeostasis thanks to the development of incubators, the assessment of adaptation to extrauterine life with the assignment of the Apgar score, the use of oxygen therapy and the development of tools for non-invasive and invasive respiratory support, the use of prenatal corticosteroids, nutritional support tools, newborn metabolic screening, the treatment of hyperbilirubinemia with phototherapy.

From the mid-twentieth century, therefore, rapid progress in the peri-neonatology field has globally led to the improvement of clinical care for the physiological and pathological term newborn and above all for the preterm newborn, allowing for a progressive reduction in infant mortality worldwide.

Preterm birth, which occurs before 37 weeks of gestational age, affects approximately 15 million babies worldwide (Harrison MS, et al. 2016) and represents the main direct cause of neonatal mortality (36% of cases), in addition to increasing infant mortality from indirect causes, with rates inversely proportional to gestational age (Lawn JE, et al. 2010; 2016) and consequently to birth weight. The improvement in perinatal and neonatal care has allowed a drastic increase in the survival of very low birth weight (VLBW <1500 g) and extremely low birth weight (ELBW <1000 g) infants, and the gestational age correlated with a survival rate of 50% decreased from 29 weeks in 1960 to 24 weeks in the early 1990s and even to 23-24 weeks in the 2000s; however, the increased survival at lower gestational ages in turn correlates with an almost stable incidence of disability as a result of the same prematurity (Glass HC, et al. 2015), with modest variations in the incidence with regard to the major or minor sequelae.

The other leading cause of neonatal mortality and infant morbidity is represented by perinatal asphyxia with consequent hypoxic-ischemic encephalopathy (Liu L, et al. 2016), which mainly affects term neonates and is often associated with a various degree of cognitive or motor impairment resulting from the brain damage (proportional to the type and duration of hypoxia). Worldwide, perinatal asphyxia accounts for 23% of neonatal deaths, with regional differences and higher incidence in low-resource countries; between the years 2000-2012, deaths from intrapartum complications decreased by about a third (from 8.2 to 5.3 per 1000 live births) (Lawn JE, et al. 2016), but it is not fully known whether, despite the improvements in the perinatal-neonatal care, the cases of hypoxic-ischemic encephalopathy have really reduced in recent years (Ferrari F. 2021). With regard to long-term outcomes, in fact, in

recent decades a reduction in major outcomes has been observed, such as infantile cerebral palsy (ICP) both in the preterm VLBW and in the moderate and late preterm infants (MPT and LPT, i.e. born to GA 32 -36^{+6} weeks), but not in the full-term nor in the preterm ELBW infants (Sellier E, et al. 2016).

1.2 From “neonatal semeiology” to “precision neonatology”

In light of the overexposed epidemiological trends and with the aim of further improving the short and long-term outcomes of both preterm and full-term infants at risk, increasing attention in neonatology is focused on understanding the more subtle physiopathological mechanisms underlying the prematurity-related diseases and the perinatal adverse events, and contextually on the study of the individual diversification of these mechanisms.

To date, scientific evidence has shown that different environmental conditions experienced early from intrauterine life and in the immediate postnatal period can profoundly affect biological mechanisms and therefore have a significant impact on future health, even in the long term. Many studies have now confirmed the Barker hypothesis and the "fetal origin of adult diseases", according to which events that occur in the early stages of fetal development have a significant impact on the risk of chronic diseases in adulthood; in particular, poor growth and fetal nutrition, the marker of which is represented by low birth weight, are correlated with the risk of insulin resistance, obesity, hypertension and coronary heart disease (Barker DJ. 1990; 1992; 1995). The "thrifty phenotype hypothesis" assumes that the epidemiological association between inadequate nutrition and growth in the fetus and in the first years of life and the onset of metabolic syndrome in later ages is explained by permanent changes in glucose and insulin metabolism (Hales CN , Barker DJ. 2001); in particular, the long-term effects of low birth weight seem accentuated by subsequent (compensatory) excessive growth, the biological basis of which seems to be represented - among the various mechanisms - by a high rate of cell divisions with shortening of telomeres, that accelerates the processes of cell death. There is therefore a "phenotypic plasticity" that allows any given genotype to implement biological variations aimed at adaptation in evolving environments (Barker DJ, et al. 2002), the result of which may however be harmful over time for the same organism. Epigenetic modifications represent the molecular substrate of the interaction of environmental factors, more or less modifiable, with non-modifiable genetic factors. These modifications (DNA methylation, post-translational modifications of histones, non-coding microRNAs) are involved in the physiology of development; when resulting from exposure to specific environmental events in early stages of life that represent the window of greatest plasticity, they nevertheless contribute at the same time to the definition of individual susceptibility to specific diseases (Feinberg AP. 2007). Exposure to endogenous or exogenous factors in critical prenatal-postnatal periods therefore determines structural and/or functional changes that affect developmental plasticity in a potentially permanent way, through a gene dysregulation that can also be transmitted to the next generations.

These evidences explain the continuous and ever growing interest in the concepts of fetal programming and developmental reprogramming or imprinting (Tang WY, et al. 2007; Gicquel C, et al. 2008). Fetal

"*programming*" and "*reprogramming*" can be linked to a number of different perturbations in the maternal compartment, such as alterations in maternal nutrition and reduced utero-placental blood flow, and the central role of the placenta is therefore evident (Jansson T , Powell TL. 2007; Nugent BM, et al. 2015). Telomere length represents a potential marker of intrauterine fetal programming events; initial telomere length setting and telomerase expression capacity or activity can be influenced by suboptimal intrauterine conditions through various mechanisms involving maternal-placental-fetal oxidative, immune, endocrine and metabolic pathways (Whiteman VE, et al. 2017; Entringer S, et al. 2018). Hypoxia represents a basic mechanism involved in pregnancy disorders and complications of fetal and neonatal development, mediating changes in the fetal growth trajectory and modulating gene expression through epigenetic mechanisms (Fajersztajn L, Veras MM. 2017).

These biological substrates explain in a broad sense the importance of the mother-placenta-fetus/newborn triad in achieving long-term global health. In light of these adaptive paradigms aimed at establishing phenotypes that meet the needs of the future life environment but at the same time predispose to the risk of disease, the importance in obstetrics, perinatology and neonatology of defining individual risk categories and stratifying the risk for prognostic purposes is evident.

A large amount of scientific literature documents the transition, in just over a century, from the realization of the existence of the newborn as a patient (first of obstetricians, then of pediatricians and finally of neonatologists) to the appropriation of the concepts of "*precision medicine*" and "*individualized tailored medicine*" (Goetz LH, Schork NJ. 2018; Williams JR, et al. 2019), which in the neonatal setting find one of the main potential fields of application, considering the impact of early interventions on the future health of individuals with the maximum theoretical life expectancy.

The current challenge of medicine, including neonatal medicine, is in fact represented by the development of personalized care solutions according to a tailored approach, that is, tailored to the individual patient; this in turn justifies the growing need for a critical analysis of perinatal data aimed at the "benchmarking" process: the observation and contextualization of increasingly detailed data contributes to the definition of individualized strategies aimed at the progressive improvement of clinical performance. Perinatal medicine and precision neonatology can therefore be interpreted as the repetition of continuous cycles of evaluation of the mother-placenta-fetus/newborn triad, which lead to an increasingly precise stratification and therefore to the definition of specific data profiles that correlate with certain clinical endpoints, including long-term ones. In the transition from classical medicine based on anamnesis and semeiology to the current one of big data, the significance and clinical usefulness of biomarkers are easily understood.

1.3 The role of biomarkers: from bench to bedside

1.3.1 Definitions, characteristics and functions

A biomarker is a biological observation that replaces and ideally predicts a clinical endpoint of interest or an outcome that is more difficult to observe or measure, with the advantage therefore of being simpler,

faster and less expensive than direct measurement of the final clinical endpoint; to understand its value, it is necessary to know the physiopathological relationship between the biomarker and its clinical endpoint (Aronson JK, Ferner RE. 2017). The World Health Organization (WHO) defines a "biomarker" as “any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease” (WHO. 2001). In accordance with the BEST (Biomarkers, EndpointS, and Other Tools) Resource, a glossary containing definitions relative to biomarkers and useful tools for the development of medical products aimed to a personalized medicine, a biomarker is “a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions” (FDA-NIH Biomarker Working Group. 2016), and can be represented by the dosage of macro-molecules (DNA, RNA, proteins, lipids), cells or processes that describe a normal or abnormal biological state in an organism. The use of biomarkers at a clinical level is preceded by a long process that begins with the discovery or identification of a potential biomarker (a prerequisite of which is the knowledge of the physiopathological mechanisms underlying a specific process or condition of interest, and possibly the mechanisms through which a certain intervention modifies the process itself), to the evaluation of its accuracy and to the subsequent evaluation of the clinical impact ("validation") (Aronson JK, Ferner RE. 2017; Hayes DF. 2015).

An ideal biomarker should be characterized by biological validity, high sensitivity and specificity, high positive and negative predictive values, reproducibility, low cost, as well as being simple to measure and repeatable. The uses of biomarkers can be manifold, from disease screening to providing prognostic information and evaluating response to therapeutic interventions. Indeed, there are susceptibility/risk, diagnostic, monitoring, prognostic, predictive, response (pharmacodynamic and surrogate endpoint), and safety biomarkers, depending on the purpose and clinical use (FDA-NIH Biomarker Working Group. 2016; AIFA 2014).

A large amount of scientific works testifies to the continuous efforts in researching new biomarkers, with the ultimate goal of a medicine that is as individualized as possible. After an exponential increase starting from the 2000s, in 2011 over 850,000 publications related to the keyword "biomarkers" were reported in Pubmed, although only about a hundred biomarkers actually validated and effectively used in clinical practice corresponded to these (Poste G. 2011). To date, over 965,000 publications are available, and many research sectors are constantly expanding, first of all the one of oncology. According to Slikker, the role of biomarkers in precision medicine is a strategic opportunity for recent technological developments to improve human health and reduce health care costs (Slikker W Jr. 2018), as the goal of precision medicine is precisely to provide every single patient the diagnosis but also the personalized treatment in order to limit waste of resources and avoidable collateral events (with a view to overall savings in economic and health terms); however, precision medicine is impossible without precision in the measurement and validation of biomarkers (Gyawali B. 2017).

1.3.2 Role of biomarkers in the peri-neonatal setting and peculiarities

To date, the identification of reliable disease biomarkers may have many potential applications in both research and clinical medicine. In the perinatal and neonatal setting, the importance of biomarkers is easily understood, considering the future life expectancy of the patient and the possibility of implementing, through their use and their prognostic indications, early interventions aimed at modulating the course of diseases and conditions that impact on the short- and long-term global health outcome.

For this reason, in recent decades the use of non-invasive laboratory biomarkers has become a key element in clinical practice, and the search for new biological markers that allow early identification of infants at risk (allowing both careful monitoring of the disease and prognostic informations) represents a strategic objective of several ongoing and ever-expanding researches (Bersani I, et al 2015) (figure 1).

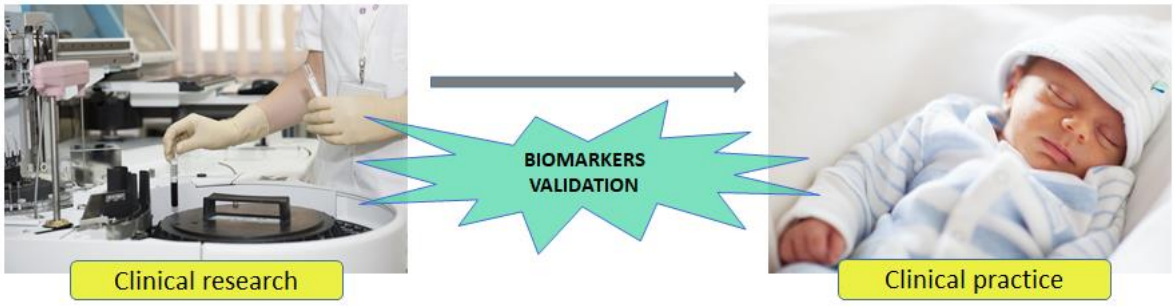

Figure 1. The biomarker management process, from clinical research to clinical practice through the validation phase

The "validation" process and the use of biomarkers in neonatal clinical practice are more complex than in other clinical areas due to some peculiarities of neonatology: age-related maturational differences, the need for different reference values for different types of newborns (e.g. term and preterm), the need to interpret and contextualize the data in many different but often interrelated clinical scenarios, and, last but not least, the choice of biological samples in compliance with fundamental requirements such as non-invasiveness and use of minimal quantities. Biomarkers must therefore meet specific requirements to be used reliably in perinatal medicine: they should be thoroughly studied in the pediatric population, measured using commercial kits available worldwide, characterized by adequate reproducibility, comparable with ranges of normality available for term and preterm infants, investigated in different biological fluids and involving a minimum discomfort related to the test (Bersani I, et al 2015; Meyer S, et al. 2017).

The samples that can be used in neonatology for the research and dosage of potential biomarkers are many: in addition to biological fluids (blood, urine, cerebrospinal fluid, meconium, saliva, lung fluid or alveolar broncholavage) of the newborn, which must be spared as much as possible, placental material, cord blood, amniotic fluid, human milk and biological fluids of the mother (blood/serum/plasma, urine) can also be used. This is particularly important for the critical preterm infant, in which a loss of 11-22 ml/kg/week (equivalent to 15-30% of the volume of circulating blood) has been demonstrated for carrying out laboratory tests in the first 6 weeks of life (Saito-Benz M, et al. 2020). In neonatology, not only the newborn should be considered, but the mother-fetus dyad and the mother-newborn dyad or,

more precisely, the mother-placenta-fetus/newborn triad, in a complex framework of reciprocal interactions that contribute to programming and developmental reprogramming (figure 2).

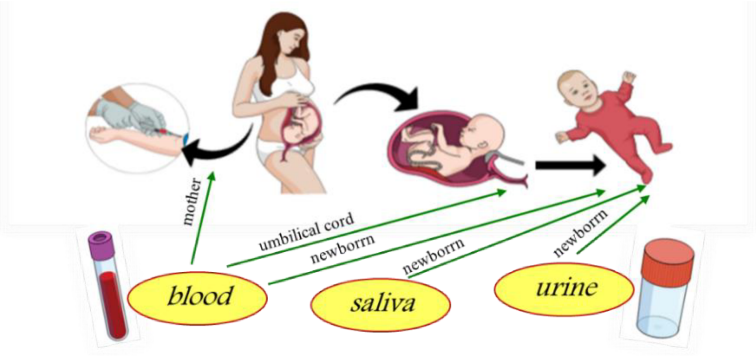

Figure 2. Biological samples usable in neonatology for the determination of biomarkers: the complex interaction in the mother-placenta-fetus/newborn triad [from Perrone S, Laschi E, Buonocore G. Oxidative stress biomarkers in the perinatal period: Diagnostic and prognostic value. Semin Fetal Neonatal Med. 2020;25(2):101087.]

1.3.3 "Conventional" biomarkers and "new" biomarkers: *from bench to bedside*

In the neonatal setting, the use of biomarkers must therefore be aimed at early identification of infants at risk of short- and long-term pathologic conditions (involving both full-term and preterm infants, the latter moreover at risk of diseases related to the same prematurity), but also aimed at monitoring ongoing non-preventable diseases and providing prognostic information about the same. The use of some biomarkers is now consolidated in neonatal clinical practice, other potentially usable biomarkers are in the validation phase, finally others are still today mainly confined to the field of research, according to a continuous evolutionary path from the laboratory to the clinical setting (from bench to bedside).

The main example of practical application of biomarkers now validated in clinical routine is represented by neonatal screening, now known as extended neonatal screening thanks to tandem mass spectrometry technology (LC-MS/MS). Newborn metabolic screening aims to identify early inborn errors of metabolism through the quantitative or qualitative evaluation of analytes, which can be primary biomarkers (i.e. specific for a specific disease, or primary condition) and at the same time can help identify other conditions (defined secondary) of clinical relevance for the patient (Donati A, et al. 2018).

Over the past two decades, neonatologists have also routinely used biomarkers to diagnose neonatal infections and sepsis, an important cause of mortality in term and preterm infants; most of these, like acute phase proteins, are non-specific immunomodulatory mediators of the inflammatory cascade characterized by varying sensitivity, specificity and diagnostic accuracy. C-reactive protein (CRP) (Hofer N, et al. 2012) and procalcitonin (PCT) (Vouloumanou EK, et al. 2011) represent the most studied and used in clinical practice, particularly useful as monitoring and pharmacodynamic biomarkers, but many other potential biomarkers are still under study (including presepsin, interleukin-6, interleukin-8, lactoferrin ..), although to date none of these individually meet sufficient requirements of diagnostic adequacy and clinical utility (Delanghe JR , Speeckaert MM. 2015; Sharma D, et al. 2018).

With advances in biochemical and genetic research, "organ and/or disease specific" biomarkers are being studied, which can be represented by protein biomarkers (such as troponin T, glial acid fibrillar

protein or protein S100) or by nucleic acids (e.g. circulating free fetal DNA or microRNAs), and the search for "new" biomarkers to support diagnosis and evaluate the prognosis of neonatal diseases using the most recent mass screening techniques, such as Next-Generation Sequencing (NGS), is constantly evolving (Ng PC, Lam HS.2012; Condrat CE, et al. 2020).

Finally, two important groups of biomarkers to date unfortunately still confined to the field of research are the biomarkers of oxidative stress (OS) and the potential biomarkers identifiable with metabolomics (the "youngest" of the omics sciences), both of which are the object of interest of this Ph.D. and will be therefore explored in the following chapters.

Oxidative stress, an inevitable consequence of life in an oxygen-rich environment and resulting from the imbalance between the production of oxidants - free radicals - and the antioxidant defenses of an organism, is now a well-known common denominator underlying many physiological and pathological conditions in the peri-neonatal period; therefore, the study of potential OS biomarkers represents a wide area of interest in the perinatal setting. Oxidative stress is a critical factor for the *fetal programming*, representing a key process that correlates an inadequate fetal growth, an impaired fetal well-being or the preterm birth with the subsequent increased risk of disease in adolescence and adulthood (Buonocore G, et al. 2017). Currently the analysis of OS biomarkers in biological fluids is used in experimental and clinical research but not yet in clinical practice, due to the complexity of the technical procedures, the lack of automation and the cost of these determinations, which have hindered their use of routine in the clinical setting. Overcoming these technical and economic difficulties poses a challenge for the immediate future, as an accurate assessment of oxidative stress would help improve the quality of neonatal care (Torres-Cuevas I, et al. 2017).

Furthermore, new frontiers in the management of the newborns (especially the critical ones) can be opened by metabolomics, a diagnostic tool based on the recognition of multiple metabolites contained in biological fluids; metabolomics marks the transition from a descriptive science to a predictive science, with the potential to translate clinical research into real clinical benefits. Metabolomics, also defined as "the new biochemistry", is in fact a holistic approach based on the systematic study of the complete set of metabolites (metabolome) contained in a biological sample; the assumption of its potential usefulness is the concept according to which the metabolic state of the individual is a faithful representation of the individual's state of health or disease. Therefore the metabolome can be considered as the phenotype that also reflects the epigenetic modifications (Mussap M, et al. 2013), and metabolomics summarizes in itself the gene-environment interactions that concur to determine the clinical phenotype of each patient since the fetal/neonatal period: it represents a sort of "identity card" of the individual in physiological or pathological conditions, with specific conditions that correlate with a specific pattern of metabolites.

AIM OF THE Ph.D. RESEARCH PROJECT

"The follow-up of the critical infant and benchmarking: the improvement of morbidity through the critical analysis of perinatal data"

Considering the progressive increase over time of "critical" infants (especially preterm) and the growing need to individualize early interventions for a long-term health gain for these patients, the research project of the present PhD was aimed at the critical review of the literature and at the search for potential biomarkers to facilitate the diagnostic-prognostic classification, the analysis and the risk stratification in the peri-neonatology setting. The critical analysis of physiopathological mechanisms and perinatal clinical data is in fact essential to the benchmarking process, which in turn contributes to the definition of individualized strategies for improving clinical and care performance. Benchmarking, a technique developed in the world of economics in the 1980s, is a process of comparing results combined with a detailed examination of the processes responsible for the results themselves; it is the component of a continuous research cycle that includes quality improvement, evaluation of its own practices and results, comparison with better performance, implementation of change and measurement of expected improvement (Walsh MC. 2003). This process in the neonatal field is essential not only for the improvement of neonatal outcomes but, through follow-up strategies, also and above all for the improvement of long-term outcomes and therefore for the variations in morbidity data; the use of risk, diagnostic, prognostic, predictive biomarkers, especially if usable through minimally or non-invasive tests, is a crucial node for clinical management and at the same time for the benchmarking process.
Considering the breadth of the topic and the rapid expansion of this field of research, the study path relative to this project had the modest purpose of laying the foundations for further and more in-depth studies that can progressively contribute to the definition of a precision neonatology. With these objectives, the Ph.D. research project has therefore been articulated on various preliminary work fronts, which can open the way to research aimed at developing a *precision neonatology* in a continuous evolution. The present thesis aims to summarize and unify the evidence-based scientific knowledge extrapolated from the literature and that obtained through the personal studies carried out, especially in the more recent field of metabolomics.

The following topics are therefore addressed and illustrated: a brief introduction on the evolution in neonatology and the role and importance of biomarkers between research and clinical practice (Chapter 1); the conditions that define neonatal risk, even those less known and in which the long-term risk is less striking but significantly impacts on social and health costs (Chapter 2); the critical review of the literature regarding biomarkers of oxidative stress, potential clinical biomarkers of diagnostic-prognostic utility in the preterm infant (Chapter 3); the possible preventive and antioxidant defense strategies in the newborn and the potential role of melatonin in preterm infants (Chapter 4); the application of metabolomics in neonatology between physiology and pathophysiology in the long-term follow-up of both full-term and preterm newborns (Chapter 5); finally, the conclusions and future

perspectives of the research theme are briefly discussed, for a possible extension of the preliminary works presented through the project of this Ph.D. (Chapter 6).

My personal scientific contributions, produced in these years of PhD and aimed at the research project itself (original articles published on international journals, abstracts and posters presented at national and international conferences, book chapter, reports: annex 1-12) are attached at the end of the thesis.

In the annex 1 a summary of this introductory part is illustrated.

CHAPTER 2

THE INFANT “AT RISK”: PERINATAL DATA, PHYSIOPATHOLOGICAL BASES AND ROLE OF THE FOLLOW-UP STRATEGIES

2.1 Classification of the newborn "at risk"

The newborn/infant "at risk" is the object of interest of clinical neonatology research because it is potentially susceptible to early interventions aimed at modifying, in a positive sense, the subsequent course of growth and development up to the outcomes in adulthood. We can therefore consider as a newborn "at risk" any newborn who presents a -variable- risk of short-term pathology (neonatal diseases, prematurity-related diseases) and, according to the well-established concepts of fetal programming and developmental reprogramming, the potential risk of developing diseases in the long term.

Chronic outcomes can be multiple, not only concerning the cardiovascular risk front but also the neurodevelopmental, cognitive, or behavioral one, or involving other organs and systems (chronic kidney damage, respiratory outcomes, sensorineural deficits).

Infants "at risk" therefore comprise preterm infants, including very preterm (VPT, with GA between 28 and 32 weeks) and extremely preterm (EPT, with GA <at 28 weeks) but also moderate and late preterm infants (MLPT , with GA 32-37 weeks) (WHO. 2018), asphyxiated infants or infants suffering from hypoxic-ischemic encephalopathy (HIE) resulting from intra-peripartum adverse events, infants suffering from genetic or malformative syndromes, or from major surgical pathologies or inborn errors of metabolism. Within these categories, it is further possible to identify a "high risk" subgroup, which will most likely develop severe acute illness or a short or long term adverse outcome, represented by preterm babies born below 30 weeks of GA and/or 1500 g of birth weight and, among full-term neonates, those affected by moderate-severe HIE, severe malformations and some metabolic diseases (Tagliabue P.2012). For the purposes of the research work of this PhD, our attention has mainly focused on the preterm infant, whose increased survival thanks to the improvement of perinatal care has raised the problem of evaluating short, medium and long-term outcomes as well as verifying the effectiveness of perinatal interventions (SIN. 2015), and above all for the clinical relevance linked to the growing number of these patients.

The identification of the newborn at risk and its potential problems represents an essential step for the implementation of a follow-up program for these newborns, and vice versa, an adequate follow-up path allows, over time, the critical analysis and the feedback of perinatal data.

2.2 Diseases of prematurity: clinical and physiopathological correlates

Preterm birth interrupts the period of dynamic development of gestation and intercepts organs and functional systems along their specific maturation trajectory. Depending on the period in which it occurs, pathological conditions can therefore be observed - more or less frequently - linked precisely to the immaturity of the organs and systems but also to the effects of the clinical interventions that are implemented to ensure the survival of the newborn and promote its development. Therefore, the

incidence of morbidity, as well as mortality, is the greater the lower the gestational age and birth weight are, in consideration of the minor fetal development that occurred in these cases precisely due to the shorter stay in the uterus. Almost all organs can be affected by an altered process of functional development and maturation and therefore by pathology, in the context of a global immaturity that makes these same diseases exclusive of the preterm population (Patel RM. 2016).

2.2.1 Short-term neonatal outcomes

The *need for resuscitation* in the delivery room is greater in the preterm infant, and inversely proportional to the gestational age. Immediately after birth, the infant must ventilate the lung to initiate the transition from fetal to postnatal circulation, but most extremely premature infants fail to ensure adequate autonomic lung expansion due to an increased chest wall compliance, respiratory muscle weakness, functional alterations of the epithelial sodium channels and surfactant deficiency. As a result, most VPT and EPT infants require positive pressure ventilation or supplemental oxygen after birth; although these supports are often necessary to ensure adequate gas exchange, they can at the same time promote acute lung injury from baro- and volutrauma and the formation of oxygen-derived free radicals (Foglia EE, et al. 2017; Tataranno ML, et al. 2015). However, even less preterm infants may need more assistance in the delivery room: Bajaj et al. found that, in a cohort of GA infants between 29 and 34 weeks, only 24% received routine care in the absence of some form - more or less intensive - of resuscitation, and that an increased resuscitation intensity in the delivery room is associated with prolonged respiratory and nutritional support, prolonged hospital stay and increased mortality (Bajaj M, et al. 2018).

Respiratory Distress Syndrome (RDS) is the main respiratory complication of preterm, due to quantitative and/or qualitative surfactant deficiency in an immature pulmonary system, particularly in the absence of antenatal steroid administration; optimal management of RDS (Sweet DG, et al. 2019) includes lung protection strategies aimed at reducing one of the most common long-term outcomes of severe and extreme prematurity, namely bronchopulmonary dysplasia (BPD) or chronic lung disease (CLD). *Patent ductus arteriosus* (PDA), a finding present at birth which usually undergoes spontaneous closure, can complicate the course of VPT newborns especially if in the presence of RDS and may require pharmacological or surgical closure, to be evaluated in based on the associated risk (both with treatment and with the PDA itself) of adverse outcomes such as intraventricular haemorrhage (IVH), BPD and death (Sweet DG, et al. 2019; Patel RM. 2016). Infections (*early-onset sepsis*, EOS occurring in the first 72 hours-7 days of life or *late-onset sepsis*, LOS occurring later >7 days) represent a relevant and potentially lethal complication in VLBW and ELBW infants, especially when they recognize a fungal or gram-negative etiology; multiple risk factors contribute to the development of sepsis in the preterm , particularly susceptible due to a combination of immune immaturity and predisposing conditions such as invasive devices and the use of broad-spectrum antibiotics in the NICUs. Prolonged premature rupture of membranes (pPROM) and chorioamniositis have been associated with the risk of EOS (Puopolo KM, et al. 2018), while the duration of parenteral nutrition to the one of LOS (El Manouni

El Hassani S, et al. 2019). In preterms who survive a sepsis episode, a correlation with subsequent growth failure and long-term neurodevelopmental sequelae is reported. These outcomes can also result from another serious complication - the most frequent gastrointestinal one - that is necrotizing enterocolitis (NEC), linked to intestinal immaturity of the preterm (deficiency of local immune defenses, altered microbiome) in combination with a series of risk factors: maternal (substance use, chorioamniositis), perinatal (intra-peripartum events leading to hypovolemia and therefore to an altered intestinal flow) and neonatal (ventilatory support, type of nutrition, use of drugs); NEC can result in surgical short bowel syndrome (Patel RM. 2016; Meister AL, et al. 2020). Another common problem of very-extremely preterm infants, represented by nutritional difficulties, contributes to its pathogenesis; in fact, these newborns usually need a prolonged nutritional support, mostly parenteral, which leads to frequent hydroelectrolytic and metabolic imbalances (such as hypo-hyperglycemia, hyperbilirubinemia, alterations in calcium-phosphoric metabolism) and increases the risk of short-term (first of all, sepsis) and long term (for example, growth failure or osteopenia) secondary complications. Finally, one of the fearful complications of prematurity is the hemorrhage of the germinal and intraventricular matrix (Germinal Matrix-Intraventricular Hemorrhage, GM-IVH), the most frequent intracranial haemorrhage linked to the rupture of the fragile capillaries of the subependymal germinative matrix (critical site of neuronal and glial precursors) and thus correlated to predisposing factors such as the instability of flow and intracranial pressure and therefore to a systemic hemodynamic instability; it usually originates in the first days of life and can variously extend into the ventricles and/or complicate with periventricular venous hemorrhagic infarction (degrees I-IV according to Volpe or Papile) (Atienza-Navarro I, et al. 2020). The more severe forms, more frequent as the GA decreases, are associated with medium-long term outcomes (evolution in post-hemorrhagic hydrocephalus, periventricular leukomalacia, motor impairment, cognitive retardation and/or neurosensorial outcomes) (Patel RM. 2016).

2.2.2 Medium- and long-term outcomes

The medium- and long-term outcomes observable in preterm infants represent the additional evolution over time of the altered maturational development resulting from prematurity itself and linked to an environment (the extrauterine one) inevitably different from the physiological intrauterine one, of the neonatal outcomes of the single patient and of the management and medical treatment of these problems, as well as "environmental" interventions including sensory and family stimulations. Similarly to neonatal ones, their incidence increases as the gestational age at birth decreases.

One of the most frequent long-term outcomes is the respiratory one, represented by *bronchopulmonary dysplasia* (BPD) or *chronic lung disease* (CLD), which is the consequence of a deviation from the normal pattern of lung development, in which prematurity itself and a series of potentially interfering factors (including genetic factors and postnatal therapeutic interventions) can play etiopathogenetic roles. Hystorically it was related to an aggressive approach to the mechanical ventilator in terms of peak pressures and oxygen concentrations on a relatively mature lung without surfactant ("old BPD"), but currently the observed pictures are attributable to the interruption of lung development with consequent

reduced alveolarization and impaired capillary development ("new BPD"), to which many factors contribute, including baro-volutrauma from mechanical ventilation, oxygen toxicity, maternal smoking, infections (Principi N, et al. 2018). The definition of BPD has been studied and modified over the years but still remains heterogeneous today: initially defined as oxygen-dependence at 28 days of life, since 2000 the diagnostic and severity definition includes the assessment of the need for oxygen and/or respiratory support at 36 weeks of postconceptional age (for infants of GA<32 weeks) or 28-56 days of life (if GA≥32 weeks) (Jobe AH, Bancalari E. 2001). BPD is associated with long-term functional pulmonary changes and it is a risk factor for post-discharge rehospitalizations, airway infections (including respiratory syncytial virus bronchiolitis), asthma-like symptoms or wheezing in the first months and years of life, and pulmonary hypertension (Principles N, et al. 2018); it also correlates with poor growth and unfavorable neurodevelopmental outcomes (Patel RM. 2016). The definition that best seems to predict BPD-related morbidity in childhood seems to be based on the assessment of the type of respiratory support at 36 weeks of postconceptional age (Jensen EA, et al. 2019).

Another significant adverse outcome of prematurity, also strictly interconnected with the presence of BPD, is the typical poor growth of preterm infants, defined as *Extrauterine Growth Restriction* (EUGR) or *Postnatal Growth Failure* (PGF). In these newborns, particularly VLBW and ELBW, the centile of weight at hospital discharge is in almost all cases lower than that of birth, and the EUGR/PGF is defined variously as a statistical measure: as a weight at 36 weeks of corrected age or at hospital discharge <10th centile (or a standard deviation score SDS < -1.28), or <3rd centile (or SDS <-2), or as a difference in Z-score between birth weight and weight at discharge >1 or > 2 (Shah PS, et al. 2006). This condition is likely due to the difficulty of implementing the ambitious growth goal proposed by the American Academy of Pediatrics in 1985, that is, maintaining in the extrauterine environment a growth rate equivalent to that one which would have been in utero if pregnancy continued until the end (AAP.1985: "achieving a postnatal growth that approximates the in utero growth of a normal fetus at the same postconception age appears to be the most logical approach [..]"). Actually, "statistical" definitions are probably not optimal for describing the real problem that can lead to long-term outcomes (Fenton TR, et al. 2020), namely a deviation of growth that correlates with an unfavorable auxological and neurodevelopmental outcome, although a longitudinal definition is preferable (Peila C, et al. 2020) and an evaluation of the growth pattern that takes into account all the auxological parameters, and not just the weight, is desirable. Furthermore, the other important feature closely related to EUGR that must be monitored over time is the altered pattern of *catch-up growth* (the linear acceleration of growth following periods of restriction, which is sometimes statistically defined as the achievement of > -2 SD of the reference population or as a variation > 0.67 SD), which in turn represents a risk factor for adverse outcome up to adulthood: while this recovery seems to correlate with a better neurodevelopmental outcome, on the other hand an excessive catch-up growth or a certain timing in which this occurs is associated with an increased risk of cardiovascular and metabolic syndrome in adulthood, confirming the Barker hypothesis (Euser AM, et al. 2008; Cooke RJ. 2012; Ruys CA, et al. 2021). The growth

pattern can also be particularly altered in preterm infants born small-for-gestational age (SGA) or after an intrauterine growth retardation (Intrauterine Growth Restriction, IUGR): it is now known that the condition of SGA is associated with a high risk to develop excessive adiposity and metabolic diseases in adulthood, in particular hypertension, increased cardiovascular mortality and type 2 diabetes mellitus (Roggero P, et al. 2011). Prematurity, especially when combined with the condition of IUGR/SGA but also in case of EUGR, is moreover associated with the risk of chronic kidney damage and kidney failure in adulthood; this supports Brenner's hypothesis according to which a reduced endowment of nephrons, with lower glomerular number and larger compensatory glomeruli, leads to an increased risk of progression in any renal disease (Gjerde A, et al. 2020; Crump C, et al. . 2019; Wand J, et al. 2009). As a consequence of nutritional and metabolic imbalances and the altered postnatal growth pattern, another complication associated with prematurity is represented by *osteopenia* (or *Metabolic Bone Disease* - MBD), characterized by hypophosphataemia, hyperphosphatasemia and late onset of radiological findings of bone demineralization which can lead to pathological fractures. Preterm infants have a low mineral reserve as most of the deposition usually occurs in the third trimester of pregnancy; furthermore, the use of steroids, inadequate enteral nutrition and prolonged parenteral nutrition, and immobilization contribute negatively to the physiological deposition (Chinoy A, et al. 2019).

With regard to neurological and neurodevelopmental aspects, *post-hemorrhagic ventricular dilatation* (PHVD), *post-hemorrhagic hydrocephalus* (post- hemorrhagic hydrocephalus, PHH) and *periventricular leukomalacia* (PVL) are fearful brain lesions due to their correlation with unfavorable neurodevelopmental outcomes - cognitive, motor, and behavioral. PHVD occurs in 30-50% of newborns with III-IV grade IVH, and differs from PHH for the absence of CSF hypertension; CSF hypertension, which precisely characterizes the condition of hydrocephalus, usually requires decompression and derivation interventions. Infants with progressive PHH requiring neurosurgery are usually at greater risk of adverse neurodevelopmental outcomes than those with stabilized PHVD (Dorner RA, et al. 2018). PVL is defined as a damage of the deep cerebral white matter (WM), distinguished in two characteristic patterns: focal periventricular necrosis and diffuse damage of the cerebral white matter. The focal form, generally cystic, is most commonly observed in the WM at the level of the watershed areas adjacent the lateral ventricles and near the foramen of Monro, border territories of the immature vascular system which is easily compromised in case of fluctuations of cerebral perfusion; diffuse WM damage is generally the overall result of perturbations in cerebral blood flow, resulting in hypoxic-ischemic loss of premielinating oligodendrocytes (Novak CM, et al. 2018). Early diagnosis is mostly neurostrumental (ultrasound and magnetic resonance imaging), while clinical diagnosis is linked to cognitive, behavioral, motor and sensorineural outcomes of which PVL is the main cause in preterm infants below 32 wks GA. *Sensory deficits* (auditory and visual deficits) can result from brain damage but also from direct involvement of the sense organs, and largely contribute to an unfavorable long-term neurodevelopmental outcome. The visual damage can be linked to *retinopathy of prematurity* (ROP), a vasoproliferative retinal disease that selectively affects the not-yet or not-fully vascularized retina of the

preterm and develops through two steps: an initial arrest in retinal vascular development resulting in microvascular degeneration, followed by an attempt of late compensation with abnormal neovascularization. A key role in the etiopathogenesis is therefore played by hypoxia-hyperoxia and by the vascular endothelial growth factor (VEGF, essential signaling protein in neoangiogenic processes) (Hartnett ME. 2017). The spectrum of ophthalmological findings of ROP ranges from minimal sequelae that do not affect vision, to bilateral retinal detachment resulting in total blindness (Casteels I, et al. 2012). The *hearing deficit* can be classified in various types (central, sensorineural, transmissive, linked to auditory dysynchrony), various degrees, progressive and/or permanent, and recognizes a multifactorial origin being influenced by genetic and non-genetic factors, such as hypoxic-ischemic lesions, infections, hyperbilirubinemia, acoustic stress in TIN, use of ototoxic drugs in early life (Zhu X, et al. 2020). Deprivation of the auditory stimulus at a later age compromises the development of language, cognitive functions and related social skills, and therefore the neurodevelopmental global outcome.

Given the many prematurity-related diseases, it is easy to understand that preterm infants have an increased risk of post-discharge *rehospitalization*, especially in the first two years of life. About 15% of preterm infants require at least one rehospitalization within the first year, with a higher readmission rate (31%) for those born <25 weeks GA; however, the highest total cost of readmission is due to the least premature infants (MLPT), which represent the largest cohort. The most common cause of re-hospitalization is represented by respiratory tract infections (Underwood MA, et al. 2007).

All the diseases potentially observable in preterm, briefly listed above, contribute to determining in turn the overall clinical outcomes that can be found at greater temporal distance and that, in their chronicity, are associated with relevant social and economic costs, i.e. neurological and neurological outcomes (see below, paragraph 2.5) and cardiovascular and metabolic problems. Among school-age children and adolescents born preterm, 35% to 50% have neurodevelopmental deficits that require special educational services; in early childhood, increased blood pressure, reduced vascular growth, signs of increased peripheral vascular resistance and cardiac remodeling may also be observed. Preterm babies, especially those with intrauterine growth restriction and neonatal acute kidney injury (AKI), are at risk for chronic kidney disease; furthermore, a reduced insulin sensitivity and a higher risk of metabolic syndrome are observed in preterm infants with excessive weight gain in childhood (Luu TM, et al. 2017). Adults born preterm have significantly higher total body fat mass, blood pressure, fasting glucose and insulin and cholesterol levels than adults born at term (Markopoulou P, et al. 2019).

2.2.3 Physiopathology of prematurity and role of oxidative stress in prematurity-related diseases

To date, the causes of labor and preterm birth are not fully known; preterm birth is a complex "syndrome" that can be induced by various factors able of triggering myometrial contractions, premature rupture of membranes and cervical maturation (infections, various maternal conditions - for example preeclampsia - or fetal conditions, use of substances and environmental factors). The molecular basis of these factors seem to be represented by a common denominator which is the activation of inflammatory

processes (Di Renzo GC, et al. 2018), closely interconnected with another - now recognized- etiopathogenetic factor which is oxidative stress (OS), namely the imbalance between the generation of free radicals and the antioxidant defenses. OS is essential in the evolution of normal pregnancy, but if excessive and persistent it determines the impairment of the antioxidant capacity and the reducing systems of the placenta; the accumulation of free radicals (FRs) and reactive oxygen species (ROS) in particular causes damage to lipids, proteins and DNA in the placental tissue with consequent premature aging of the placenta itself, placental insufficiency and fetal compromise (Sultana Z, et al. 2017). OS-induced tissue damage is the probable trigger of several downstream effects such as the onset of labor or pPROM (Menon R, Richardson LS. 2017), and generates a vicious circle of events (inflammatory cascade, cell death by apoptosis and tissue destruction) which cannot be compensated by the defense systems and ultimately leads to preterm birth, as well as - in different stages of gestation - to incorrect implantation of embryos, spontaneous abortions, malformations, IUGR and low birth weight (Menon R. et al. 2014; Toboła-Wróbel K, et al. 2020). From the very beginning of pregnancy, therefore, the fragile redox balance (reduction-oxidation) plays a fundamental role both in the physiological processes of development and growth of the fetus-placental unit and in the pathological processes responsible for pregnancy pathologies (Wu F, et al. 2015). Pregnancy itself is a state of OS, resulting from increased metabolic activity in placental mitochondria and increased production of ROS due to the greater metabolic demand of the growing fetus, but when imbalance originates from increased production of pro-oxidant factors and/or reduction of anti-oxidant factors which physiologically are responsible for the elimination of ROS ("scavenger"), cell damage occurs and causes placental and fetal pathology (Sultana Z, et al. 2017), predisposing to long-term consequences according to the theory of *fetal programming*.

This imbalance and the consequent OS-induced cell/tissue damage also occur in the newborn and particularly in the preterm infant. The fetus and the newborn are both highly susceptible to oxidative insult due to a series of predisposing factors: the overload of the aerobic metabolism linked to the increased energy demand, the presence of conditions characterized by hyperproduction of FRs (hypoxia-ischemia, mechanisms hypoxia-reperfusion, hyperoxia, inflammatory conditions, high levels of free iron, drugs), the immaturity of antioxidant defense systems; these conditions are all the more evident the lower the gestational age. Indeed, the preterm infant is not adequately prepared for the hyperoxic challenge represented by the transition to extrauterine life, that normally involves an exposure to oxygen concentrations much higher than intrauterine ones with the first breaths, and therefore a drastic increase in PaO2 (from 40-50 mmHg to 70-80 mmHg in the first 5-10 minutes of life) (Torres-Cuevas I, et al. 2017), especially if resuscitation with supplementary oxygen (FiO2> 21%) is necessary, not infrequent case in preterm births. In this way an overload of ROS occurs, and on the other hand the preterm infant is provided with a lower reserve of enzymatic and non-enzymatic antioxidants (Perrone S, et al. 2017). All these conditions contributes to cellular and tissue damage that, in the developing organism, is in turn responsible for the peculiar anomalies of prematurity-related diseases.

It is now clearly demonstrated by animal and human studies that resuscitation with oxygen-enriched air is directly toxic not only for the lungs but also for other organs such as heart, liver and brain (Saugstad OD, et al. 2012). Many of the most relevant conditions associated with prematurity, including hypoxia at birth, retinopathy of prematurity, bronchopulmonary dysplasia, intraventricular hemorrhage, periventricular leukomalacia, and necrotizing enterocolitis, have been associated with the use of oxygen supplementation and immaturity of the antioxidant defense system during the postnatal period (Torres-Cuevas I, et al. 2017; Falsaperla R, et al. 2020). The demonstration of the etiopathogenetic role of OS as a common denominator for these conditions has led to configure them, as a whole, with the term "*Free-Radicals Diseases*" (FRD) or more precisely "*free radical related diseases of prematurity*" (Perrone S, et al. 2012; Perrone S, et al. 2018; Perez M, et al. 2019) (figure 3).

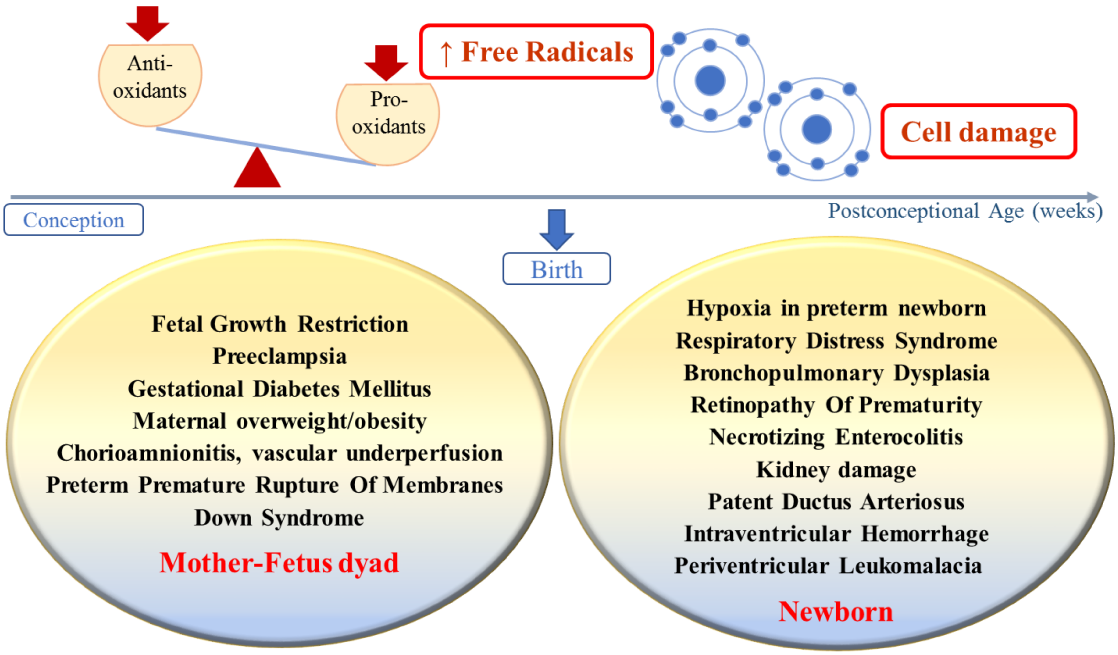

Figure 3. Adapted from Perrone S, Laschi E, Buonocore G. FRBM. 2019 (graphycal abstract)

In the energy metabolism, oxygen functions as an electron acceptor in the mitochondrial respiratory chain; accepting 4 electrons, oxygen is normally reduced to water H_2O, but this process requires four steps: each intermediate stage occurs within the mitochondria and generates ROS, including the superoxide radical O2•− (by reduction with only one electron), hydrogen peroxide H_2O_2 (by reduction with two electrons) and the hydroxyl radical •OH (by reduction with three electrons). H_2O_2 is not a free radical, despite being classified within ROS, and acts as an essential signaling molecule for cellular cross talk (e.g. for the regulation of blood flow within the arterial duct and pulmonary circulation); on the other hand O2− and •OH are free radicals, unstable molecules (possessing unpaired electrons), and therefore highly toxic as they are able to interact with cellular constituents, causing damage to cell membranes due to lipid peroxidation, protein oxidation and DNA oxidation (Perez M, et al. 2019). Under physiological conditions, approximately 98% of O_2 undergoes a complete reduction, while 2% undergoes a partial reduction with ROS production (Perrone S, et al. 2017). Mitochondria, through the enzyme NADPH oxidase (NOX) and the oxidative phosphorylation, therefore represent the main source

of ROS, but at the same time are themselves a primary target of oxidative damage, with consequent defects in oxidative phosphorylation and mitochondrial dysfunction; other sources of ROS are cytoplasmic enzymes such as xanthine oxidase (XO), nitric oxide synthase (NOS), various other oxidases present in subcellular localizations including the endoplasmic reticulum (ER) and peroxisomes, as well as various superoxide dismutases (SOD1-SOD3) that contribute to localized production of H_2O_2, and cytochromes (Perez M, et al. 2019; Sies H, Jones DP.2020). Nitric oxide (NO) can combine with oxygen free radicals, in particular superoxide anion radical, to form peroxynitrite (ONOO−), a reactive nitrogen species (RNS). ROS and RNS have a short half-life, but being highly unstable they react with neighboring molecules such as proteins, DNA, RNA, carbohydrates or free fatty acids, altering their structure and/or function. In presence of transition metals, in particular iron (Fe2 +), the generation of hydroxyl radicals is highly enhanced through the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$) (Torres-Cuevas I, et al. 2017).

Antioxidants are any substance capable of eliminating ROS and their derivatives, and can be divided into enzymatic and non-enzymatic scavengers; in particular, their functions can be classified into distinct lines of defense, according to their mechanisms of action: preventive agents that suppress the formation of new radicals, including enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), or metal-binding proteins such as ferritin and ceruloplasmin, and minerals such as selenium (Se), copper (Cu) and zinc (Zn); agents that eliminate radicals by inhibiting the initiation and/or propagation of the chain, such as glutathione, albumin, vitamins C and E, carotenoids and flavonoids; repair enzymes, that reconstitute damaged cell membranes and include lipases, proteases, DNA repair enzymes, transferases and methionine sulfoxide reductase; adaptation agents that generate appropriate antioxidant enzymes and transfer them to the essential site of action (Falsaperla R, et al. 2020). If these defenses do not counterbalance the production of FRs, cell damage occurs. *Proteins* can be damaged through oxidative modifications including the oxidation of amino acid residues containing sulfur, hydroxylation of aromatic groups, nitration of tyrosine residues, chlorination of aromatic groups or the conversion of some amino acid residues into carbonyl derivatives; *lipids* undergo peroxidation through enzymatic oxidation mechanisms (oxidation of arachidonic acid by cyclooxygenase and lipoxygenase, with formation of prostaglandins, prostacyclines, thromboxane, leukotrienes and lipoxins), but above all through non-enzymatic oxidation mediated directly by ROS, with the main target represented by the phospholipid component of biological membranes and plasma lipoproteins and in particular by polyunsaturated fatty acids (PUFA), with final formation of lipid hydroperoxides (LOOH); *DNA* undergoes various modifications including nucleotide oxidation, strand breakage, base loss and adduct formation (Falsaperla R, et al. 2020). The products of oxidative damage of cellular components can represent, as detailed below, the biomarkers of oxidative stress detectable in biological samples and therefore usable in the clinical setting (see figure 4).

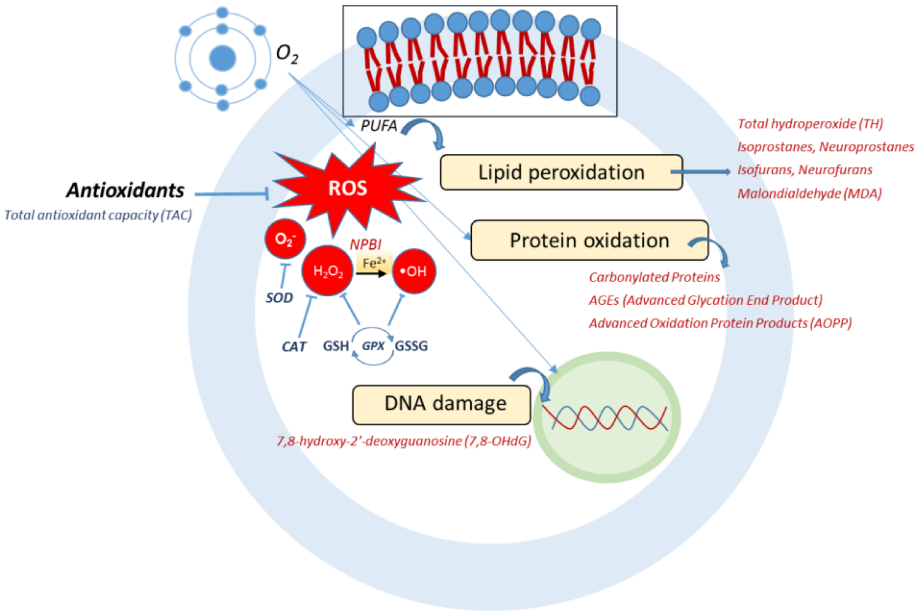

Figure 4. The products of oxidative damage of cellular components can represent the biomarkers of oxidative stress detectable in biological samples and usable in the clinical setting [from Perrone S, Laschi E, Buonocore G. SFNM. 2020.]

In the clinical practice, it has been recognized that the generation of ROS following hyperoxia is responsible for injuries affecting the lungs, the central nervous system, the retina and the red blood cells, as well as for generalized tissue damage, which can be detected both in the neonatal period and in adult life (Perrone S, et al. 2017).

At the *pulmonary* level, OS causes damage to the respiratory epithelium, inactivation of the surfactant and an inflammatory state; therefore the formation of intra-alveolar edema, interstitial thickening and subsequently fibrosis and pulmonary atelectasis ensue. These histopathological changes clinically correlate with the increase in the demand for mechanical ventilation, and the earlier and the more the preterm infants are exposed to OS, the more severe long-term respiratory outcomes can occur. Lung damage has been shown to be caused directly by ROS production in response to hyperoxia, and also indirectly due to phagocytic activation and inflammation. Phagocytic cells in the lung mediate their antimicrobial functions through the release of lysozyme, peroxidase and protease, but in addition induce further release of ROS and NO; the release of inflammatory mediators can stimulate the endothelium to produce adhesion molecules, with consequent migration of transendothelial cytokines, whose concentration increase could enter a "final common path" integrating inflammatory and oxidative pathogenetic mechanisms, that leads to lung damage: this results in impaired alveolarization, characteristic of bronchopulmonary dysplasia. Human studies have shown a quantitative increase in oxidative lipids and proteins damage in lung tissue and a reduction in antioxidant levels in biological fluids of ventilated preterm infants (Perrone S, et al. 2017; Perrone S, et al. 2018; D'Angelo G, et al. 2020). Annex 3 below represents a personal in-depth analysis on the role of oxidative stress in the etiopathogenesis of bronchopulmonary dysplasia (Chapter 16 in: Springer Nature Singapore Pte Ltd. 2019. Sajal Chakraborti et al. (Eds.), Oxidative stress in lung disease (Vol-1)).

At the *brain* level, the pathogenesis of damage is complex and linked to multiple events, but even in this case the first events in the cascade leading to brain lesions are attributable to inflammation and oxidative damage due to the production of FRs: ROS can initiate the release of pro-inflammatory cytokines and the activation of microglia, with a consequent release of other FRs and pro-inflammatory molecules (D'Angelo G, et al. 2020). In particular, H_2O_2 and nitric oxide radicals (NO•) activate the soluble enzyme guanylate cyclase, that catalyzes the formation of cyclic "second messenger" guanosine monophosphate (cGMP); cGMP modulates the function of protein kinases, ion channels and other important targets, resulting in abnormal dilation of the arterioles, greater fluid filtration, leukocyte obstruction of capillaries and release of inflammatory mediators and platelet activation. The oxidative events that trigger the initiation of bleeding in the germinal matrix promote a cascade leading to the breakdown of tight junctions, increased permeability of the blood-brain barrier, and activation of microglia in developing periventricular WM: these events are mediated by cytokines (IL-1β and TNF-α), VEGF and NO, but also activated microglia release ROS, which in turn increase endothelial damage, alter haemostasis and increase anaerobic metabolism. The progression of endothelial dysregulation contributes to the progression of IVH (Perrone S, et al. 2018).

At the *retinal* level, OS plays a key role in the pathogenesis of ROP. In phase I of vascular attenuation, the arrest of normal retinal vascularity is driven by hyperoxia, as in response to hyperoxia the developing retinal endothelial cells activate various transcription factors, including HIF-1α and VEGF, that in turn determine the cessation of the growth of the retinal vessels and the loss of some existing retinal vessels; furthermore, hyperoxia generates ROS due to the lack of a self-regulating retinal blood flow system that acts across a narrow perfusion pressure range. In fibrovascular proliferative phase II, hypoxia stimulates vascularization but an abnormal retinal vascular proliferation occurs with the formation of a ridge, which exerts traction on the retina and increases the risk of detachment; hypoxia induces the formation of ROS mainly through NOX, but also induces the activation of NOS with a consequent increase in nitric oxide reacting with the same ROS, and so generating nitrites, nitrates and peroxynitrites, causes of retinal microvascular damage (thus the so-called "nitro-oxidative stress"). In both phases of the ROP, therefore, VEGF plays an important role but in the opposite way; on the other hand, OS acts continuously through the generation of intracellular ROS, that act as signaling effectors (Perrone S, et al. 2017; Perrone S, et al. 2018; Graziosi A, et al. 2020).

At the *intestinal* level, OS contributes to the pathogenesis of NEC, a multifactorial disease. In preterm infants ischemic intestinal events are frequent and favored by systemic hypotension, hypothermia, PDA, anemia, especially in the case of increased mesenteric needs after the start of enteral feeding; ischemia leads to endothelial cell dysfunction, with alteration of the endothelin1/nitric oxide balance in favor of vasoconstriction, loss of electrons from the mitochondrial electron transport chain and increased availability of redox-active transition metals, resulting in ROS formation. Thus, the damage of cell membranes from lipid peroxidation and oxidative damage to proteins and other macromolecules is achieved, as well as an up-regulation of the inducible nitric oxide-synthase (iNOS), with consequent

overproduction of NO that, together with O2− and through the Haber-Weiss reaction catalyzed by iron, leads to the formation of peroxynitrites, contributing greatly to cell damage. OS also causes the partial inactivation of cyclooxygenase-1 (COX-1) thus reducing the generation of protective prostaglandins (Pg), and the concentration of GPX, an important antioxidant enzyme, also appears to be reduced. All these mechanisms cause significant inflammation of intestinal tissues, the release of inflammatory mediators and the down-regulation of cell growth factors; the proinflammatory cytokines in turn activate a cascade of events leading to the eventual breakdown of the intestinal mucosal barrier and in some cases to a severe NEC (Perrone S, et al. 2014; Perrone S, et al. 2018).

At the *erythrocyte* level, damage derived from free radicals contributes to the pathogenesis of neonatal hemolytic anemia, in particular in preterms; after exposure of red blood cells to OS, there is a rapid loss of activity of age-dependent enzymes by reticulocytes (SOD, CTX, GPX) probably due to proteolysis; in addition, prolonged exposure to oxygen causes changes in the shape of erythrocytes, as a consequence of the toxic effects of oxygen on erythrocyte membranes (Perrone S, et al. 2017). The release of iron resulting from hemolysis (free iron) itself contributes to the OS through the Fenton reaction (Perrone S, et al. 2018).

Finally, OS also contributes to the pathogenesis of damage at the *renal* level. FRs-mediated lipid peroxidation has been implicated as a mechanism of tissue damage during ischemia; the products of lipid peroxidation directly influence renal function by causing renal vasoconstriction or decreasing the glomerular capillary ultrafiltration coefficient and therefore the glomerular filtration rate (Perrone S, et al. 2018).

2.3 The very - extremely preterm "high risk" infant

As mentioned above, the shorter the time spent in utero and therefore the lower the gestational age at birth and the greater is the degree of immaturity of the organs and consequently the risk of prematurity-related diseases, given the greater susceptibility to the physiopathological damage linked to the aforementioned mechanisms of oxidative stress and inflammation and the greater probability of ROS formation due to the hyperoxia to which VLBW and ELBW infants are exposed.

According to the Vermont Oxford Network (VON), the leading international network that collects data on preterm <1500g and/or <30 weeks of EG, in 2017 RDS was diagnosed in about 80% of preterms born at 28 wks GA and up to 90% in those born at 24 weeks, requiring surfactant administration in total 55% of VLBW infants. BPD has been coded for 18% of VLBW infants in Europe (Sweet DG, et al. 2019). PDA is a common finding in EPT, found in 32-60% of preterms 22-28 weeks GA (Patel RM. 2016). EOS have an incidence of 6 cases per 1000 births <34 weeks, 20 per 1000 births <29 weeks, and 32 cases per 1000 births between 22 and 24 weeks; However, recent studies report a decrease in the EOS rate from 19-32 cases per 1000 in the 1990s to the current 9-11 cases per 1000 VLBW births (Puopolo KM, et al. 2018). NEC represents the most common and fearful gastrointestinal complication of EPT, affecting 1 in 10 births with a high mortality estimated up to 30-40% in ELBWs (Patel RM. 2016). IVH occurs in about 31% of preterms <27 weeks who undergo ultrasound screening; in the

French cohort EPIPAGE 2 (Etude Epidémiologique sur les Petits Ages Gestationnels 2), it is reported that about 20-30% of those born with GA <29 weeks have a GMH-IVH or intraparenchymal haemorrhage, one third of whom develop PHH requiring insertion of a shunt in 10-20% of cases (Gilard V, et al. 2020). Severe IVH (grade 3 and 4) occur in 38%, 26%, and 7% of surviving infants >12 hours born at 22, 24, and 28 weeks of gestation, respectively (Novak CM, et al. 2018), and is associated with an increased risk of all adverse neurodevelopmental outcomes (Patel RM. 2016). Among VLBW and/or born <30 weeks, over 40% develop some stage of ROP; most regress without treatment but approximately 12.5% (almost exclusively <1250 g) progress to severe ROP (Quinn GE, et al. 2018). The real incidence of osteopenia of prematurity is not known, given the lack of validated dagnostic criteria, nor is the impact of the long-term consequences known: it has been suggested that the changes observed in infants or young adults could precede the development of osteoporosis early in later adult life (Harrison CM, Gibson AT.2013). Annex 4 illustrates a work (abstract presented at the XXVII National Congress of the Italian Society of Neonatology), carried out with the aim of evaluating bone mineral density in young adults born preterm, assessed at an average age of 20 years: no significant differences were found between young adults born at term and preterm as regard ultrasound evaluation absolute values, but a difference was found in preterm SGA compared to term infants; furthermore, a significant association between bone ultrasound values and lipid profile of young adults born preterm has been demonstrated, further confirming the impact of fetal programming on health outcome in later ages.

Overall, mortality in infants born between 22 and 24 weeks of GA is 64% and survivors have a 43% probability of presenting a varying degree of neurodevelopmental impairment (Ream MA, Lehwald L. 2018). According to the EPIPAGE-2 study, 0.7% of those born before 24 weeks of gestation survive until discharge: 31.2% of those born at 24 weeks, 59.1% at 25 weeks and 75.3% of those born at 26 weeks. Survival rates are 93.6% from 27 to 31 weeks, and discharged infants without severe neonatal morbidity represent 0% at 23 weeks, 11.6% at 24 weeks, 30.0% at 25 weeks , 47.5% at 26 weeks, 81.3% between 27 and 31 weeks (Ancel PY, et al. 2015).

2.4 The moderate - late preterm “at risk” infant

Moderate and late preterm infants represent over 80% of all preterms; although at a lower risk of significant diseases than VPT and EPT, their number justifies their impact in terms of health, social, and economic costs. Preterm birth interrupts a period of dynamic development and intercepts organs and functional systems along their specific maturation trajectory, from this it follows that even the last 6 weeks of gestation can represent a critical window for development, in particular of lungs and brain (Kugelman A, Colin AA.2013). Therefore, also in the MPT and LPT population for each week of GA different and specific functional skills can be observed; this explains the increased incidence of adverse neonatal outcomes observed in this group. Although in 80% of cases these infants have a neonatal course without particular complications, when compared with their term counterparts they show an increased risk of pathologies related to their developmental immaturity: need for resuscitation maneuvers at birth,

feeding difficulties, jaundice, hypoglycemia, thermal instability, apnea, transient tachypnea/respiratory distress, bacterial infections. The result is an increased incidence of diagnostic tests for sepsis and administration of antibiotics, intravenous infusion of fluids, various modalities of respiratory support, and increased hospital length of stay after birth (Kugelman A, Colin AA.2013). The incidence rates of these complications are inversely related to gestational age, so they decrease with the progress of the GA during the period between 32 and 37 weeks. Overall, LPT infants have a 3.5 times higher neonatal morbidity rate during hospital stay and a 4.6 times higher mortality rate than those of full-term newborns, as well as an increased risk of hospital readmission in the neonatal period and in the first year of life. (Engle WA, et al. 2007; Gouyon JB, et al. 2012). Compared with term counterparts, LPTs present a greater risk of adverse short-term outcomes such as RDS (RR Relative Risk 17.3), IVH (RR 4.9) and neonatal mortality (RR 5.9), and beyond the neonatal period a greater risk of infant mortality (RR 3.7) and ICP (RR 3.1) (Teune MJ, et al. 2011). MPTs understandably have intermediate morbidity and mortality rates between LPTs and VPTs, while receiving less attention than the latter (Gouyon JB, et al. 2012). According to the EPIPAGE-2 study, overall survival rates are 98.9% and newborns discharged home without severe neonatal morbidity still represent 96.8% of those born between 32 and 34 weeks (Ancel PY, et al. 2015) . However, it is increasingly demonstrated that the risk of long-term adverse outcomes is also increased in MLPTs compared to full-term infants, especially with regard to respiratory, auxological and neurodevelopmental outcomes.

With regard to lung development, preterm exposure to the extrauterine environment, hyperoxic compared to the fetal one, is a condition that interferes with normal development even in the final stages of gestation and therefore can play a role in the subsequent development of pulmonary dysfunction; furthermore, at 34 weeks the lung volume is equal to 47% of the final volume, and normally the further increase in the exchange surface occurs until the end (Kugelman A, Colin AA.2013; Colin AA, et al. 2010). Edwards et al described increased incidence rates of respiratory disorders in preterm infants up to 10 years of age, with odds ratios as higher as the GA decreases at birth, but with an increased risk in the MLPT category of 33-36 weeks (Edwards MO, et al. 2016).

With regard to brain development and therefore the possibility of neurological and neurodevelopmental outcomes, between 20 and 40 weeks of gestation the weight of fetal brain increases by 90% in an almost linear manner (at 34 weeks it is equal to 65% of that of the brain at term of pregnancy); the volume of SG increases linearly (+1.4% or +15 ml/week) with a consequent 4-fold increase in cerebral cortical volume, and the volume of myelinated WM shows a dramatic 5-fold increase between 35 and 41 weeks; synaptogenesis, dendritic arborization and axonal elongation are also incomplete and actively proceed not only until the end of gestation but also beyond the neonatal period (Kinney HC.2006). It follows that even the CNS of the MLPT is still immature and therefore potentially vulnerable to insults that interfere with the processes of neuronal and glial maturation and development, such as those responsible for PVL; the potential underlying molecular mechanisms include, also in this GA range, the vulnerability of oligodendroglial precursors, the damage induced by glutamate and that mediated by cytokines and

free radicals, in addition to the lack of maturation-dependent antioxidant mechanisms (Kugelman A, Colin AA.2013; Kinney HC. 2006). This can explain the greater risk of long-term neurodevelopmental problems in MLPTs, variously considered -in the different studies- as difficulties or deficits in cognitive, sensorineural, motor, socio-emotional, neurobehavioral and educational fields (see below in detail, paragraph 2.5).

Finally, long-term auxological and metabolic problems can also be observed in these newborns. In MLPTs, IUGR is more common, as it may itself cause preterm birth, and even the most mature infants are vulnerable to feeding difficulties that put them at risk of poor weight gain and growth failure in early childhood (Goyal NK, et al. 2012). Any subsequent catch-up growth of suboptimal quality determines differences in the body composition of these infants compared to their term counterparts, with preferential early deposition of fat mass to the detriment of the lean one ("catch-up fat") (Giannì ML, et al. 2012; 2016): these alterations predispose in later age to increased adiposity that, in association with a hyper-responsiveness of adipose tissue and hyperinsulinemia, can represent risk factor for the development of metabolic syndrome in young adulthood.

The annexes 5 and 6 (poster presented at the XXVII National Congress of the Italian Society of Neonatology on 26-29 September 2018 and on the 3rd jENS - Congress of joint European Neonatal Societies held in Maastricht on 17-21 September 2019) illustrate the results of two works carried out by the undersigned on the cases of preterms of 32-36 weeks GA born at the University Hospital in Siena in the years 2016-2017, aimed at investigating respectively the neonatal outcomes and the auxological outcome in the first year of life in the comparison groups of MPT and LPT infants. Our observational studies confirm that these (apparently) lower risk newborns, and in particular those born at 32-33 weeks GA, can present many clinical problems, in the short term and potentially at in the long term, with a significant clinical, social and economic impact.

2.5 Long-term neurodevelopmental outcomes

Over half of preterm infants who survive the neonatal age experience minor developmental problems at school age, while about 10% develop true cerebral palsy (CP), sometimes complicated by sensorineural problems (Ferrari F, et al. 2017) . As illustrated above, VPT/VLBW infants represent the high risk category for brain damage, from which long-term developmental problems can arise, such as PCI, motor and cognitive difficulties, visual and hearing impairments, behavioral problems, relationship, socialization and emotional difficulties. Neuroanatomically, VPTs have an overall reduced brain volume, particularly in the frontotemporal regions and in the hippocampus, especially in the presence of predisposing postnatal factors such as sepsis, BPD, IVH, steroids, oxygen therapy and sedation; however, also MLPTs have a reduction in the overall size of the brain, corpus callosum, deep GM and cerebellum, as well as a reduced myelination upon reaching the term. MRI studies have allowed us to observe that preterm infants have a slower maturation than the full-term counterparts at the level of the corpus callosum, inferior longitudinal fasciculus and temporal-occipital visual pathways. Differences in total brain volume and size of various brain regions persist into adolescence and adulthood and the

intelligence quotient (IQ) appears to be related to the size of these specific regions (Ream MA, Lehwald L.2018). Furthermore, selective damage to the sense organs is additional to the sensorineural damage, configuring a visual and/or auditory deficit which in turn contributes to unfavorable neuro-evolutionary outcomes. These are represented by a broad spectrum of pathology associated with prematurity: several studies have found that preterm infants are at increased risk for psychiatric problems such as autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), anxiety, and depression; motor abnormalities; cerebral palsy (CP); sensory abnormalities such as problems related to vestibular balance, pain processing, blindness and deafness; delays in different areas of development (language, cognitive, sensory and motor development); worse school performance than age-matched full-term infants (Hee Chung E, et al. 2020). Adverse neurodevelopmental outcomes can therefore manifest themselves in various ways and in different periods of life (from the first months to infancy, preschool and school age, up to adolescence and adulthood), also depending on the type and degree of the underlying brain damage, the brain areas involved, the environmental factors and the timeliness of diagnosis and enabling interventions. For example, neuromotor disabilities, especially gross motor disorders or CP, can be suspected early from the first months of life, allowing early targeted interventions to be implemented; instead, neuropsychological disabilities (meaning by neuropsychological functions all the components that make up the overall intelligence of an individual, namely language, memory, attention, executive functions, visual perception and visual-motor integration skills), in most of the cases are not diagnosable in the first two years of life as many of these functions and skills mature at later ages; finally, the disturbances of the emotional-relational sphere and of psychic functions can also manifest themselves in later ages, from adolescence to adulthood, and this is easily understood taking into account that neurological development is a gradual and complex process in which the CNS reaches its "adult" configuration around the age of 30 yrs and it is in any case susceptible to neurobiological changes, even later (Ferrari F, et al. 2017).

Cerebral palsy and motor disorders: CP is one of the major disabilities and the most severe motor outcome observed in the preterm infant, often associated with other types of deficit; CP is defined as a group of permanent disorders of the development of movement and posture, with consequent limitation in daily activities, caused by non-progressive damage of the developing brain of the fetus or newborn, and is often accompanied by sensory, cognitive, communication disorders, behavioral problems or epilepsy (Bax M, et al. 2005). The prevalence of CP increases with decreasing GA, and it is estimated to be 14.6% (95% CI, 12.5-17.0) in children born EPT (22-27 weeks), 6.2% (95% CI, 4.9% -7.8%) in VPT infants (28-31 weeks) and 0.7% in MLPT (32-36 weeks of gestation) compared to 0.11% (95% CI, 0.09-0.14) in full-term infants (Himpens E, et al. 2010); however, a decreasing trend in CP prevalence in VPTs is currently described (Sellier E, et al. 2016; Adams-Chapman I, et al. 2018). Preterm infants are more likely to have bilateral CP, and spastic diplegia is the most frequent subtype (Ream MA, Lehwald L. 2018); CP cannot be diagnosed before a few months or even before 2 years of age, however some early movement and posture anomalies (general movements, GMs) suggest early the evolution in

CP (Ferrari F, et al. 2017). However, today minor disabilities such as transient dystonia and postural instability in the first months, or subsequently the coordination disorders (Developmental Coordination Disorders, DCD) and clumsiness or Minor Neurological Dysfunctions (MND) that can affect tone, posture or manipulation are more frequently observed (Ferrari F, et al. 2012).

Neurosensory deficits: visual function plays a decisive role in normal psychomotor development; preterm infants have a high risk of developing visual disturbances, linked both to peripheral visual system deficits (ROP, consequent refractive defects) and to the neurological damage that is configured with the term of Cerebral Visual Impairment (CVI) and corresponds to the malfunction or damage to the visual pathways, i.e. optical radiation, occipital cortex and associative visual areas (Philip SS, Dutton GN. 2014; Ferrari F, et al. 2017). Similarly, normal auditory input is an essential requirement for the development of auditory perception and therefore for the development of language; preterm babies have an increased risk of auditory disability, related to hearing impairment (understood as impairment of the hearing threshold) and hearing deprivation with failure to develop neural networks in the stages of auditory plasticity (SIN 2015), mainly due to coexisting factors such as exposure to ototoxic drugs, brain damage, hearing stress from NICUs, possible infections (Zhu X, et al. 2020). Such sensory dysfunctions, if present, therefore contribute to the adverse neuromotor and cognitive outcome of preterms (especially EPTs).

Language delay: language development and communication require a complex interplay of auditory, social, motor and cognitive skills and depend on the social environment in which the infants grows up; language acquisition begins from the first moments of life, and therefore is a domain that can be compromised by preterm birth, even in the absence of severe brain damage, and can have long-lasting effects (SIN. 2015; van Noort-van der Spek IL, et al. 2012). Among infants born below 26 weeks GA, 25% have speech disorders in preschool-age, but overall in those born <36 weeks GA a third is affected. Neuroimaging studies have made it possible to observe that functional connectivity is altered in the pathways involved in language, and that these alterations persist into adulthood and correlate with linguistic scores (Ream MA, Lehwald L. 2018).

Cognitive and neuropsychological disorders: a high percentage of VLBWs (about 30-50%), even in the absence of evident or severe brain damage, develop various forms and degrees of cognitive problems, probably subtended by lesions of the WM only detectable with functional neuroimaging studies (Ferrari F, et al. 2017); the lower the GA and the lower results the intelligence quotient (IQ) of children born preterm. In school age, about 30% of VLBWs and 40-50% of ELBWs have learning difficulties that require specific support: this does not seem to be related only to an IQ deficit but rather to a more global impairment of neuropsychological functions, that may be more or less individually concerned (Aarnoudse-Moens CS, et al. 2009; Litt JS, et al. 2012). The assessment of the cognitive sphere already in preschool age (3-5 years) allows a classification of related disorders and therefore the start of enabling treatments that enhance the possibility of recovery of the deficient functions, thanks to still active neuroplasticity processes (Ferrari F, et 2017). The most frequently reported problems in VPTs-EPTs

(but also possible in less immature infants) include: intellectual disability (historically has been described an IQ in VLBW-ELBW infants on average 3-9 points lower, with a decrease of 0.3-0.6 SD, compared to their term counterparties; Aylward GP. 2002); impaired attention, especially reduced selective visual attention (present in 38% of 5-year EPTs versus 6% of term controls) and executive function disorders (Orchinik LJ, et al . 2011); visual-perceptual and visual-motor integration disorders (with significantly lower scores in the tests of manual dexterity and graph-motor deficits; Lind A, et al. 2011); memory disorders (present in 24-34% of ELBWs compared to 6-9% of term infants; Orchinik LJ, et al. 2011). Given the complexity of cognitive functions, closely interrelated, recent studies are aimed at delineating the longitudinal neuropsychological profile of preterm infants, in order to identify clinical biomarkers that allow a diagnosis and therefore the implementation of interventions as early as possible: for example, in an Italian study the 1-year Griffith Scale scores were predictive of total, verbal and performance quotients of the 7- and 10-year WPPSI and WISC scales; this also confirms the importance of the relationship between the various development profiles, as the primarily motor one in the first year of life is closely interconnected with emotional development in the first two years (playing a central role in the possibilities of exploration and autonomy), and both contribute to the overall cognitive development assessable at 3 years and the subsequent development of logic and reasoning skills observed at 5 years (Squarza C, et al. 2017; Ferrari F, et al. 2017).

Behavioral problems and disorders of the relational and emotional sphere: behavioral problems include a broad spectrum of psychic, behavioral, emotional and social disorders (including conduct problems, mood disorders, relationship problems with peers and emotional difficulties, psychosomatic problems). Several authors have described a "preterm behavioral phenotype" that includes a tendency to internalize traits (anxiety, depression), inattention and social difficulties (Johnson S, et al. 2011); externalizing behaviors, including aggression, are also more common in preterms (Arpi E, et al. 2013). According to Hornman, all preterm infants (including the more mature MLPTs) compared to term infants have higher rates of persistent (7.2% vs 3.6%), emerging (4.3% vs 2.3%), and resolving (7.5% vs 3.6%) emotional and behavioral problems; in VPT-EPTs the rates of persistent and emerging problems are higher, while the MLPTs more frequently have emotional-behavioral resolving problems (Hornman J, et al. 2016). The problems encountered in preterm infants can therefore vary according to age (both for onset and for their duration), but the behavioral disorders detected in the first years of life generally find a continuity in school age and adolescence (Ferrari F, et al. 2017). Overall, the prevalence of ASD, ADHD and anxiety disorders is higher in VPTs; predisposing risk factors include perinatal factors (length of NICU stay, steroid use, WM injury) (Spittle AJ, et al. 2009) and social factors (mainly dysfunctional mother-infant interaction patterns and maternal mental health) (Muller-Nix C, et al. 2004; Ferrari F, et al. 2017). The Annex 7 at the end the thesis illustrates a personal work (published in Brain & Development), that was carried out at the University Hospital of Siena on young adults born preterm, aimed at evaluating their personality, emotional and cognitive functions, and testing the hypothesis that these subjects may have cognitive and emotional consequences related to prematurity that persist into adulthood, not

observable in young adults born at term. Fifty-five young adults born preterm (mean age 18.58 ± 2.42 years; born <33 weeks of gestational age and/or with birth weight <1500 grams) were enrolled. The verbal IQ (Verbal Intelligence Quotient, vIQ), the performance IQ (Performance Intelligence Quotient, pIQ) and the total IQ (Total Intelligence Quotient, tIQ) were assessed using the Wechsler Adult Intelligence Scale - Revised (WAIS-R); personality profiles were studied using the Rorschach test. Both WAIS-R and Rorschach scores were subsequently compared with 13 full-term controls. The data was analyzed with the SPSS v20 statistical package for Windows.

In our study, young adults born preterm showed lower IQ scores than full-term young adults: tIQ 90.95 ± 22.46 vs 108.77 ± 16.14, p = 0.006; vIQ 89.85 ± 21.85 vs 107.69 ± 18.33, p = 0.009 and pIQ 92.40 ± 22.90 vs 108.31 ± 14.52, p = 0.011. There was no difference in the personality profile as most of the subjects showed adequate internal resources in both groups, but a greater tendency to anxiety and insecurity was identified in young adults born preterm.

Overall, young adults born preterm show greater psychological fragility and lower cognitive patterns than young adults born at term; these data support those present in the literature regarding the need for an early psychological intervention that can help these subjects at greater risk to face a society that is changing and that necessarily requires stronger internal resources.

2.6 Role of the follow-up strategies

Identifying the high-risk infants is the key step in implementing a follow-up program for these same infants; follow-up is essential for many reasons, and it is easily understood in the light of the above. Primarily, it is a need from the point of view of clinical care, as these patients, especially survivors with BPD/CLD or brain injury, require special surveillance of growth and development; in addition, the surveillance of long-term outcomes is necessary to monitor the overall quality of care of each perinatal center (with a view to benchmarking). The results to be examined must include growth, the presence of signs suggestive for cerebral palsy and/or its diagnosis, the evaluation of sensorineural impairment and cognitive functions, but also the identification of minor neurological disorders. It follows that the minimum requirements for the follow-up of high-risk infants are the periodic assessment of growth and neurodevelopment and sensory development during the first 2 years of life. However, an ideal program should include all aspects of care, including assessment of outcomes, social and educational intervention, and medical and habilitation treatments, even for longer periods and hopefully through school age and adolescence (Tagliabue P .2012), taking into account the potential problems, even minor ones, that preterm infants face, particularly if born at the lowest GA. Especially with regard to neurological and neurodevelopmental outcomes, a structured follow-up program is therefore essential to allow early diagnoses, that in turn are essential to implement targeted interventions when neuroplasticity is still at its full potential (Ferrari F, et al. 2017); however, follow-up programs must include a global assessment of the prematurity-related future health risk, also taking into account the cardiovascular and renal risks that arise later but in which pre-perinatal and postnatal factors (especially EUGR followed by excessive catch -up growth) can play a key role. Preventive strategies feasible during

the most susceptible developmental period (up to term-correct age and during the first twyears of life) can mitigate the long-term negative consequences of preterm birth, and such strategies need to be established on an individual and social level (Chehade H, et al. 2018). If the follow-up program is aimed at categories of subjects at greater risk, the minimum target is represented by preterm infants born below 28-30 weeks GA and/or with a birth weight <1000-1500 g; if the follow-up has a primary clinical-care objective, it is necessary to evaluate case by case, that is, including all subjects with risk factors and/or a potentially developmental clinical history (SIN. 2015). As mentioned above, even the most "mature" LPTs have a higher frequency of neurodevelopmental outcomes and an increased metabolic and cardiovascular risk, which make long-term clinical monitoring desirable and necessary even for these infants; however, their large number compromises the inclusion of all these newborns in the follow-up networks (Favrais G, Saliba E.2019), making it even more necessary identifying the subjects at greatest risk among all categories of newborns (to include and follow over time, regardless of gestational age).
Further research must be focused on the physiopathological mechanisms underlying long-term outcomes, on the identification of early risk biomarkers and on potential therapeutic targets, including epigenetic ones, to better define individual risk profiles and facilitate early prevention (Chehade H, et al. 2018). Therefore, alongside the clinical setting, during the entire care path from birth to later ages (therefore during the stay in neonatal intensive care unit and over the subsequent follow-up), biomarkers can be useful and essential for risk classification, diagnosis, and definition of prognosis of every individual patient. For this reason, a critical review of the literature regarding potential biomarkers of oxidative stress, a key physiopathological mechanism in prematurity-related diseases, will be presented in the next chapter.

CHAPTER 3

CRITICAL REVIEW OF OXIDATIVE STRESS BIOMARKERS IN PERI-NEONATAL CLINICAL RESEARCH

3.1 Oxidative stress biomarkers

Perinatal oxidative stress (OS) is an inevitable consequence of life taking place in an oxygen-rich atmosphere, and occurring when the production load of FRs is not adequately countered by intracellular antioxidant systems. The fetus and the newborn are highly susceptible to oxidative insult, due to the presence of multiple predisposing factors that determine a hyperproduction of FRs (hypoxia-ischemia, hypoxia-reperfusion mechanisms, hyperoxia, inflammatory conditions, high levels of free iron, drugs) and at the same time a reduction of antioxidant defenses. Already during the entire period of gestation, OS plays a key role in placentation, in maintaining homeostasis (but - by contrast - also in altering homeostasis) of the fetal-placental unit and the intrauterine environment, and in fetal programming, through regulation of gene expression and modulation of cell growth (Perrone S, et al. 2016). Oxidative damage is particularly accentuated in preterm infants, who are often exposed to high concentrations of oxygen starting from birth and then from resuscitation in the delivery room, with consequent increased production of ROS (Saugstad OD, et al. 2012; Tataranno ML, et al. 2015). OS is therefore largely responsible for cell, tissue and organ damage in the perinatal period, so much so as to contribute, as explained above, to the determination of prematurity-related diseases, that hence can be classified as OS-related from a physiopathological point of view. (FRD; Perrone S, et al. 2012; Perrone S, et al. 2018; Perez M, et al. 2019). Alongside the role played in short-term outcomes, OS also represents the general underlying mechanism that links an altered placental function to fetal programming and therefore to the origin of adult diseases, with consequent long-term impact: the role played in the fetal programming has been hypothesized to last, in the preterm infant, in the postnatal period (Perrone S, et al. 2016; Buonocore G, et al. 2017) (when the extrauterine environment of the NICU replaces the physiological intrauterine one).

For these reasons the study of potential OS biomarkers represents a wide area of interest in the neonatal field, since the identification of reliable biomarkers in the perinatal period seems to be an essential prerequisite for the early diagnosis of FRDs and the consequent long-term health outcomes; however OS is difficult to measure in vivo, because reactive oxygen species (ROS), radicals concentrated on nitrogen or reactive nitrogen species (RNS), and FRs have a very short half-life and are therefore difficult to detect and measure. The general analytical methods available to study oxidative damage can be divided into two categories: those aimed at detecting the potential risk of oxidative stress, such as non protein bound iron due to its ability to generate hydroxyl radicals (•OH) through the Fenton reaction (and therefore constitutes a susceptibility/risk biomarker), and those aimed at detecting the effects of OS and therefore the oxidative damage of cellular components, i.e. oxidation in lipids, proteins and DNA (these damaged components can represent diagnostic, prognostic, pharmacodynamic, monitoring,

predictive, or safety biomarkers). Furthermore, stress response proteins, ROS-forming enzymes such as xanthine oxidase (XO), NOS and NOX, or the activity of antioxidant enzymes such as SOD, CAT and GPX can be used as biomarkers of OS.

Due to the multiple effects of OS, not a single biomarker related to a single disease or condition is described in most studies, but rather a panel of biomarkers that together can help detecting a risk condition or fetal-neonatal pathology. Unfortunately, currently, the analysis of OS biomarkers in biological fluids is used only in experimental and clinical research but not in clinical practice, due to the complexity of the technical procedures, the lack of automation and the cost of these determinations, as well as to the not infrequent difficulty of interpreting the biochemical data in the light of clinical data; however, overcoming these technical and economic difficulties is increasingly impossible to defer, considering the potential diagnostic-prognostic value of OS biomarkers and the ever-increasing technological potential available (Torres-Cuevas I, et al. 2017). The main biomarkers of OS studied and their biochemical-clinical correlation with pregnancy, fetal and neonatal pathologies are presented in the annexes 8 and 9, through a critical review of the literature (reviews published respectively in Free Radical Biology and Medicine-Annex 8 and in Seminars in Fetal and Neonatal Medicine-Annex 9).

3.1.1 Non protein-bound iron (NPBI)

Iron (Fe), a highly reactive element, is a critical component for the generation of FRs, being a strong biological oxidant and reducing agent; in particular, iron catalyzes the formation of highly reactive • OH starting from hydrogen peroxide in the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH-$. In moderate quantities and bound to proteins, it is an essential element for growth and for all aerobic metabolic processes, but it is toxic when unbound. Physiologically, iron is safely sequestered in transport proteins such as transferrin and lactoferrin and stored in proteins such as ferritin and hemosiderin. Iron ions do not exist in plasma, and the term non-protein bound iron (NPBI) was introduced to indicate a low molecular mass form of iron, free from high-affinity binding to transferrin; in this form, iron is available to react with reduced oxygen intermediates and to generate ROS, that in turn are able to release even more iron by mobilizing it from ferritin (Emerit J, et al. 2001). Therefore, the toxicity of iron is inversely proportional to the availability of ferritin necessary to sequester and make the ferrous ion non-toxic, and directly proportional to the amount of H_2O_2 available to produce hydroxyl radicals through the Fenton reaction (Tataranno ML, et al. 2015). Erythrocytes have been the first cells to reveal neonatal susceptibility to oxidative stress: OS, in fact, leads to the oxidation of hemoglobin and damage to erythrocyte membranes (Bracci R, et al. 2002). Asphyxia and acidosis predispose to an increase in the free iron content of erythrocytes; its release is accompanied by the oxidation of membrane proteins and the appearance of senescent cells antigen, representing one of the main ways of removal of erythrocytes. In neonates, NBPI release into erythrocytes is related to plasma NBPI level: released iron has a tendency to diffuse from erythrocytes into the surrounding medium (plasma), resulting in the appearance of detectable plasma NBPI (Comporti M, et al. 2002). In hypoxic infants, increased plasma NBPI concentration significantly correlates with the severity of brain injury and impaired neurodevelopmental

outcomes up to the second year of age (Buonocore G, et al. 2003). NPBI appears to be a reliable index of brain damage, reaching 100% sensitivity and specificity at high concentration. The significant positive correlation between free iron and the number of nucleated red blood cells in cord blood, currently considered a reliable index of enduring intrauterine asphyxia, suggests that the rate of erythropoiesis and the extent of NPBI are related to the degree of asphyxiation and the probability of subsequent neurological deterioration (Perrone S, et al. 2002). Furthermore, a presumed interrelation between NPBI and white matter lesions in preterm hypoxic infants has been advanced: supporting a relationship between iron and PVL, there is the observation that many weeks after IVH and PHVD the NPBI levels in cerebrospinal fluid are markedly increased (Savman K, et al. 2001), and this increase cannot be explained by hemolysis alone. Reliable measurements of NPBI are possible in studies on oxidative stress under experimental and clinical conditions, through a method based on the preferential chelation of NPBI by an excess of the low affinity nitrilotriacetic acid (NTA) ligand (Paffetti P, et al. 2006).

3.1.2 Lipid peroxidation products: isoprostanes (IsoPs), isofurans (IsoFs), neuroprostans (NPs), neurofurans (NFs); malondialdehyde (MDA)

In the presence of FRs, biological membranes (containing a relatively high percentage of polyunsaturated lipids) become susceptible to oxidation. The peroxidative damage induced by FRs to cell membrane is a potentially critical event that, if not interrupted, under certain conditions causes irreversible cell damage, initiating or promoting the pathogenesis of injury or disease states. Lipid peroxidation is a radical process whereby the polyunsaturated fatty acid (PUFA) contained in the phospholipids of cell membranes undergoes a reaction with oxygen, producing lipid hydroperoxides (ROOH). This reaction occurs through a chain mechanism initiated by the abstraction of a hydrogen atom from the PUFA by a reactive free radical, followed by a complex sequence of propagative reactions. Hydroperoxides are the major initial molecular products of lipid peroxidation and can be measured in plasma by various techniques. *Total hydroperoxide* (TH) represents an overall measure of OS, because it is indicative of intermediate oxidative products of lipids and peptides; the damage to lipids and proteins from exposure to FRs leads to the generation of lipid hydroperoxide from lipids and the formation of carbonyl and protein hydroperoxide from proteins. The lipid and protein hydroperoxides, in the presence of traces of free iron, produce several species of secondary reactive radicals, that can be collectively measured as organic hydroperoxide. Due to the rapid degradation in vitro, an accurate measurement of hydroperoxides is very difficult. The fact that the origin of lipid peroxidation products cannot be directly demonstrated represents a significant problem with lipoperoxidation tests; this limitation can be overcome by measuring a series of compounds similar to prostaglandins, called isoprostanes (IsoP) and isofurans (IsoF), produced independently of the COX pathway and whose formation is due to the oxidation of arachidonic acid (AA) and docosahexaenoic acid (DHA).

F2-isoprostanes are products similar to Pg, which come from the in vivo and in vitro peroxidation of AA and phospholipids; they are not produced by COX but only through the reactions of free radicals. F2-IsoPs are initially formed in phospholipids and then released into the blood; these prostanoids are less reactive and unstable than other peroxidation products such as aldehydes or peroxyl radicals, so they can be easily measured in plasma and urine using methods such as gas chromatography coupled with mass spectrometry (GM-MS), chromatography liquid coupled with MS (LC-MS) and immunoassays (Tataranno ML, et al. 2015). Normal adult humans have been found to have stable plasma levels of F2-isoprostanes; compared to adults, plasma F2-IsoP levels of neonates are significantly higher and an inverse relationship between IsoPs levels and gestational age has been reported (Comporti M, et al. 2004), suggesting that lipid peroxidation is already active in the prenatal period and decrease during the last gestational weeks and throughout the postnatal life. A recent study made it possible to determine the reference levels of F2-IsoP in newborns (Longini M, et al. 2017). An oxygen insertion step diverts the intermediates from the IsoPs pathway to form other compounds, called *isofurans* (IsoFs), which contain a tetrahydrofuran ring; due to this differential formation process, it has been observed that oxygen tension can influence the lipid peroxidation profile (Solberg R, et al. 2012). Like IsoPs, IsoFs are chemically stable, so they can act as in vivo biomarkers of oxidative damage; furthermore, the IsoFs/IsoPs ratio provides information on the relative oxygen tension at the time when lipid peroxidation occurs.

DHA is an important component of neuronal membranes, and its levels in the brain increase during development and decrease with aging; DHA oxidizes both in vitro and in vivo to form compounds similar to F2-IsoP called *F4-neuroprostans* (F4-NP): NPs are therefore biomarkers in vivo of selective oxidative damage for neurons. An alternative oxidation pathway of DHA leads to the formation of IsoFs-like compounds called *neurofurans* (NFs). Quantitative evaluation of NF in vivo reveals a modulated formation in conditions of increased or reduced OS, and, given the abundance of DHA in the brain, the analysis of NF may have particular value in the quantitative evaluation of lipid peroxidation in brain damage (Buonocore G, et al. 2010).

Malondialdehyde (MDA) is another lipid peroxidation biomarker frequently used in clinical trials. MDA is one of the main and most studied low molecular weight end products, highly cytotoxic due to its ability to rapidly bind proteins or nucleic acids. The thiobarbituric acid reactive substance method (TBAR test) has often been used to assess MDA concentrations, but lacks specificity; furthermore, there is a risk of underestimating lipid peroxidation (therefore has a low sensitivity) since MDA can form Schiff bases or cross-link bonds with lysine and arginine from proteins in vivo. MDA is a secondary oxidation product and also does not derive solely from polyunsaturated fatty acids, so the interpretation of MDA levels and response to the TBA test in lipid peroxidation studies requires caution (Liu J, et al. 1997). Recently, the use of ultra-high resolution liquid chromatography MS (UHPLC-HRMS) for the quantification of free and total plasma MDA using dinitrophenylhydrazine (DNPH) as a derivatizing agent has been validated (Mendonça R, et al. 2017).

3.1.3 Protein Oxidation Products: Carbonyls and Advanced Protein Oxidation Products (AOPP)

Oxidative damage to proteins is difficult to assess due to the number of different potential protein targets and amino acid residues. FRs can indeed modify the amino acid residues of proteins leading to cross-linking, changes in conformation and loss of function, but it is probable that, physiologically, the proteins damaged by oxidation are rapidly removed by the proteases rather than accumulate to easily detectable levels.

Carbonylated proteins

During protein oxidation, carbonyl groups (-CO = O) are introduced into the side chains of proteins; in fact, when proteins react with the hydroxyl radical, an abstraction of a hydrogen atom from the protein polypeptide occurs, with the formation of a radical centered on the carbon, which reacts readily with the dioxygen to form peroxyl radicals under aerobic conditions. The side chains of all amino acid residues are susceptible to oxidation by the action of ROS. In particular, this "carbonyl" stress involves lysine and arginine, and lead to the production of Advanced Glycated End Products (AGEs) such as pentosidine, a group of heterogeneous molecules resulting from non-enzymatic reactions of reducing sugars with amino groups of lipids, DNA and especially proteins (Singh R, et al. 2001). In the presence of transition metals, oxidative cleavage, the loss of histidine residues, cross-links of bi-tyrosine, introduction of carbonyl groups and the formation of alkyl, alkoxy and alkylperoxy radicals occurs; iron can bind proteins to specific sites and the resulting complex reacts with H2O2 via the Fenton reaction to supply ROS (Marzocchi B, et al. 2005). The concentration of carbonyl groups, generated through many different mechanisms, is a good measure of ROS-mediated oxidation of proteins due to their relative early formation and relative stability. Several assays are available for detecting protein carbonyls; highly sensitive methods involve derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which leads to the formation of a stable product, dinitrophenylhydrazone, easily detectable by various methods (spectrophotometric assay, enzyme-linked immunosorbent assay - ELISA and one-dimensional or two-dimensional electrophoresis followed by Western blot immunoassay) (Dalle-Donne I, et al. 2003; Weber D, et al. 2015). The assays for the dosage of AGEs are mainly based on the use of specific antibodies or on spectrofluorometric measurements based on their fluorescent properties (Marrocco I, et al. 2017). An increase in carbonyl groups has been demonstrated in the first 6 hours after a hypoxia-ischemic event in animal models (Mueller-Burke D, et al. 2008); furthermore, albumin carbonylation has been highlighted in newborns with high levels of NPBI and an unfavorable neurodevelopmental outcome (Marzocchi B, et al. 2005), in whome the altered proteins act as a trap for the FRs, that trigger further chain reactions worsening brain damage.

Advanced Oxidation Protein Products, AOPP

Since plasma proteins are the prime target of free radicals, the detection of advanced oxidation protein products (AOPP) in biological fluids may be an optimal strategy for detecting and estimating the degree of oxidant-mediated protein damage. AOPPs are the terminal products of protein exposure to the FRs,

without having themselves oxidizing properties. They include protein aggregates via disulfide bridges and/or tyrosine cross-linking. AOPPs can be measured using spectrophotometry on a microplate reader as described by Witko-Sarsat and colleagues (Witko-Sarsat V, et al. 1996), thus they represent a marker of the degree of protein damage in OS; however, current methods suffer from poor reproducibility due to lipid precipitation in plasma samples. Hanasand et al proposed a novel method that determines lipid solubilization prior to spectrophotometric measurement of AOPP levels, in order to prevent both lipoprotein loss due to precipitation and overestimation due to light scattering (Hanasand M, et al. 2012). AOPP levels are elevated in hypoxic infants, especially preterm (Buonocore G, et al. 2000; 2002).

3.1.4 DNA oxidative damage biomarker: 7,8-hydroxy-2'-deoxyguanosine (7,8-OHdG)

7,8-hydroxy-2'-deoxyguanosine (7,8-OHdG) is a reliable and frequently used marker of OS, in particular of OS-related DNA damage, because it is a guanosine-based oxidation product, namely an oxidized nucleoside released after the repair of damaged DNA. 7,8-OHdG can be detected in tissues and blood samples. Since oxidative DNA lesions, such as oxidized nucleosides and bases, are reasonably soluble in water and excreted in the urine without being further metabolized, urinary 7,8-OHdG is considered an important biomarker of generalized and cellular oxidative stress (Nakajima H , et al. 2012). This biomarker can also be detected in human peripheral leukocytes, using a high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS / MS) method (Wu D, et al. 2017; Torres-Cuevas I, et al. 2017).

3.1.5 Oxidized/Reduced Glutathione Ratio (GSH/GSSG)

Glutathione (GSH) is a tripeptide which represents the most abundant non-protein intracellular thiol, and acts as an antioxidant system thanks to its ability to eliminate ROS through reversible oxidation to its GSSG disulfide (thanks to GPX which catalyzes the following reaction: $2GSH + H_2O_2 \rightarrow 2H_2O +$ GSSG), which in turn can be reduced to GSH by the activity of glutathione reductase (GR) and the reducing power of NADPH. GSH levels and the GSH/GSSG ratio, usually between 30 and 100, decrease in case of OS. The measurement of GSH, GSSG and their relationship is considered an index of the redox status and therefore a useful marker of human diseases (Marrocco I, et al. 2017). They can be measured in biological fluids through various methods such as spectrophotometry, HPLC, capillary electrophoresis, nuclear magnetic resonance and mass spectrometry (Pastore A, et al. 2003), although known methodological artifacts reduce their potency. Recently, a Spanish group has developed reliable methods for obtaining accurate and reproducible determinations, especially useful in the neonatal period requiring minimal blood volumes (Escobar J, et al. 2016; Sánchez-Illana Á, et al. 2018).

3.1.6 ROS-generating enzymes: xanthine oxidase (XO), myeloperoxidase (MPO)

Some ROS-generating enzymes are normally present in cells and involved in various physiological functions, but they can also be responsible for an excessive oxidative load depending on the levels of their substrates and the counterpart cellular antioxidant defenses. *Xanthine oxidase* (XO) catalyzes the oxidation of xanthine to uric acid and is a well known source of superoxide radical O2−. The enzyme exists in two forms, an oxidase XO (that oxidizes xanthine to uric acid using O2 and producing O2-,

and hypoxanthine to xanthine forming H_2O_2) and a dehydrogenase XDH (which performs the same reaction using NAD+): under hypoxic conditions, XDH is rapidly released into the circulation and converted into XO, with subsequent amplification of ROS production (Frijhoff J, et al. 2015; Marrocco I, et al. 2017). *Myeloperoxidase* (MPO) is a hemoprotein or peroxidase that catalyzes the reaction between H_2O_2 and chloride ions to produce ROS, producing hypochlorous acid (HOCl) as the primary oxidant. Oxidizing species derived from MPO lead to the generation of specific oxidation products, such as 3-chloro-tyrosine (3-Cl-Tyr), an AOPP that can be used as a biomarker. A limitation of the use of MPO as a biomarker is that current methods are not standardized across laboratories and do not provide direct information on MPO activity (Frijhoff J, et al. 2015; Marrocco I, et al. 2017).

3.1.7 Enzymes with antioxidant action: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX)

Superoxide dismutase (SOD) is the main cell detoxification enzyme and most potent antioxidant, acting as a component of the first line defense system against ROS; it catalyzes the dismutation of the superoxide anion O2− into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), making the superoxide anion potentially less dangerous. There are different isoforms of SOD, all of them with the characteristic of having a redox transition metal active in the catalytic site necessary for the dismutation reaction: copper-zinc superoxide dismutase (SOD1), with copper and zinc in its catalytic core, and located in the cytoplasmic compartments intracellular; manganese superoxide dismutase (SOD2), which plays an important role as an antioxidant enzyme in mitochondria; iron superoxide dismutase (SOD-3), localized in the extracellular space. The activity of SOD can be measured analyzing the inhibition in the reduction rate of a tetrazolium salt from O2- generated through a xanthine/XO enzyme system (Vives-Bauza C, et al. 2007). SOD also acts as a pro-oxidant producing H_2O_2; therefore, other antioxidant enzymes such as CAT and GPX are required to maintain the redox balance and an imbalance in their ratio can be dangerous (Marrocco I, et al. 2017).

Catalase (CAT) is a common antioxidant enzyme, mainly localized in peroxisomes but absent in mitochondria, that catalyzes the conversion of H_2O2 into H_2O and O_2; its enzymatic activity can be measured by various colorimetric/spectrophotometric assays (Vives-Bauza C, et al. 2007).

Glutathione peroxidase (GPX) is an important selenium-dependent intracellular enzyme that breaks down H_2O_2 into H_2O and the lipid hydroperoxides into their corresponding alcohols mainly in the mitochondria, through the oxidation of GSH to GSSG; GPX activity can be measured using cumene hydroperoxide and GSH as substrates (Ighodaro OM, Akinloye OA. 2018; Marrocco I, et al. 2017).

3.1.8 Total Antioxidant Capacity (TAC)

Non-enzymatic antioxidant capacity or Total Antioxidant Capacity (TAC) is defined as the moles of oxidants neutralized by one liter of body fluids. In plasma, non-enzymatic antioxidants include endogenous and exogenous nutritional compounds, such as bilirubin and thiols on the one hand and tocopherols, ascorbic acid, carotenoids and phenols on the other (Marrocco I, et al. 2017). Various analytical methods have been developed to measure TAC, including the 2,2'-azino-bis-3-

ethylbenzothiazoline-6-sulfonic (ABTS) radical scavenging assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, ferrin reducing antioxidant power (FRAP) and the oxygen radicals absorbance capacity (ORAC); the principle of conventional methods of TAC dosage, widely used for their characteristics of ease and speed, is based on the elimination of radicals and the redox potential of antioxidants. The ABTS test appears to be an appropriate method for measuring overall plasma antioxidant capacity and predicting the body's antioxidant status in humans (Lee SG, et al. 2017).

3.1.9 Visfatin

Visfatin, also known as nicotinamide phosphoribosyl transferase (NAMPT), is a ubiquitous adipokine secreted by visceral fat, or rather a multifunctional molecule that can act intracellularly and extracellularly as adipokine, cytokine and enzyme. In recent years, visfatin has been described as a potent marker of inflammation and dysfunction and has been associated with oxidative stress (Moschen AR, et al. 2007), although its physiopathological role in humans remains largely unknown. Visfatin can be evaluated with the enzyme-linked immunosorbent assay (ELISA) and has recently been studied as a new biomarker of OS in preterm infants (Marseglia L, et al. 2016).

3.1.10 MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are a group of small non-coding RNAs known to play a unique role in post-transcriptional gene regulation and important in the regulation and modulation of physiological and pathological processes, including cellular responses to redox imbalance. MiR-200 family members play a crucial role in OS-dependent endothelial dysfunction, as well as in vascular complications of diabetes and obesity; moreover, several miRNAs, such as miR-210, have been shown to play a key role in mitochondrial metabolism, modulating ROS production and the sensitivity to them. Recent studies have revealed that a number of miRNAs act on placental development: several miRNAs are expressed specifically in the placenta, plausibly influencing the pathological responses resulting from OS (Rudov A, et al. 2014). Taking this premise into account, microRNAs circulating in maternal blood can be considered potential biomarkers for fetal hypoxia in utero and therefore useful for the detection of hypoxia in the intrapartum period (Whitehead CL, et al. 2013).

3.2 OS biomarkers in pregnancy diseases and of the fetal-placental unit: clinical-laboratorist correlates

A fragile redox balance must exist to allow for proper growth and development in pregnancy; an adequately controlled production of reactive species is a physiologically essential factor. Since the beginning of pregnancy, placentation is a process closely related to OS: ROS/RNS influence the development of the placenta itself and, at the same time, an abnormal placentation can lead to excessive OS and adverse consequences (Wu F, et al. 2015). Although OS is therefore a necessary feature of normal pregnancy and normal fetal development, oxidative overload can give rise to various pathological conditions and this can be demonstrated through the concentration of OS biomarkers in biological fluids and tissues (Cuffe JS, et al. . 2017).

One of the most studied complications of pregnancy is *Intrauterine Growth Retardation* (IUGR) or *fetal growth restriction* (FGR). By measuring IsoPs, OS has been shown to occur early in pregnancy and can be detected by dosing specific biomarkers in the amniotic fluid of high-risk pregnancies with IUGR; the dosage of isoprostanes-F2 in the amniotic fluid represents a reliable evaluation of fetal oxidative stress (Longini M, et al. 2005). Indeed, FGR is related to placental insufficiency, impaired blood flow to the fetus and intrauterine hypoxia; these conditions, when chronically maintained, evoke placental and fetal responses in the form of growth adaptation to hypoxia, hence intrauterine hypoxia can induce the generation of fetal FRs and OS. Due to the lack of fetal but also maternal antioxidant systems (Rodríguez-Rodríguez P, et al. 2018), excessive ROS production during the intrauterine period leads to a pro-oxidative state that impairs fetal growth (Dennery PA. 2010). Other biomarkers of OS, such as MDA, have been detected elevated in maternal plasma, umbilical cord plasma and placental tissues of patients with IUGR compared to the control group without IUGR, confirming the role of OS in fetal growth alterations (Biri A, et al. 2007; Kamath U, et al. 2006).

Preeclampsia (PE) is a potentially life-threatening pregnancy complication characterized by maternal hypertension, proteinuria, possible multi-organ failure and death; it is a closely related condition and potential cause of FGR. A complex correlation between OS, systemic inflammation, and vascular dysfunction underlies the condition: higher concentrations of superoxide (as a measure of systemic OS) and markers of systemic inflammation were found in pre-eclamptic pregnancies compared to normal physiological pregnancies (Mannaerts D, et al. 2018). There is irrefutable evidence of placental OS in cases of early-onset preeclampsia, including increased concentrations of protein carbonyls, lipid peroxides, nitrotyrosine residues, and DNA oxidation products (Burton GJ, Jauniaux E.2011; Myatt L, Cui X. 2004 ; Burton GJ, et al. 2009). Free F2-IsoPs were significantly higher in preeclamptic women than in normotensive controls, both in the placenta (Brien M, et al. 2017) and in maternal plasma samples (Bilodeau JF, et al. 2015). Furthermore, oxidative damage of placental DNA has been related to pre-eclamptic pregnancies with restriction of fetal growth, as demonstrated by increased levels of 7,8-OHdG in placental trophoblast cells (Fujimaki A, et al. 2011; Kimura C, et al. 2013).

Another common complication of pregnancy is *gestational diabetes mellitus* (GDM), known to be associated with overproduction of ROS and OS. Indeed, numerous evidences have revealed an increase in biomarkers of lipid peroxidation, such as the levels of MDA in maternal plasma (Al-Shebly MM, Mansour MA.2012) and the levels of isoprostanes in the placenta (Coughlan MT, et al. 2004) of diabetic women during the pregnancy. Recent studies have confirmed the higher pro-oxidant status of women with GDM by dosing higher maternal, cordonal and placental levels of XO, MDA and 8-isoprostane, and lower antioxidant defenses than in women without GDM (Shang M, et al. 2015 ; 2018). In contrast to these results, Ramírez-Emiliano J. and colleagues (Ramírez-Emiliano J, et al. 2017) recently dosed lipid peroxidation products (quantified with TBARS) and lower carbonyl levels in placentas with GDM than those quantified in normal placentas; these data seem to suggest that placentas with GDM are more protected from oxidative damage, but the same Authors admit that the difference may be related to a

better metabolic control of their patients than those in the previous GDM-OS study. Moreover, these results, although in conflict with the previous ones, are relevant because they suggest that good dietary control may be crucial to prevent the increase in oxidative damage caused by GDM.

A similar concept could be applicable and desirable in cases of *maternal overweight or obesity*, because an increase in OS characterizes both obesity and gestation. An increase in F2-IsoPs levels in SGA infants born from overweight or obese mothers has been shown (Negro S, et al. 2017), as well as high maternal levels of MDA, carbonylated proteins, NO and superoxide anion and reduced antioxidant defenses in case of obesity; therefore, changes in the redox balance were found in the placenta and infants of obese mothers, indicating a high oxidative load (Malti N, et al. 2014). As obesity in women is an increasing public health problem and FGR (possible complication of maternal obesity) is strongly associated with metabolic syndrome in adults, more careful prenatal and perinatal monitoring is increasingly needed (Chiavaroli V, et al. 2009; Alcala M, et al. 2018).

Increased levels of OS biomarkers have been detected in other conditions related to altered placental function, such as *chorioamnionitis* (CA) or *vascular hypoperfusion* (VU) associated with preterm labor: recently, significantly increased levels of IsoPs, NPBI and AOPP have been found in umbilical cord blood in cases of preterm CA and VU (Perrone S, et al. 2016), confirming the complex interrelation between OS and the functioning of the fetal-placental unit, with inevitable repercussions on the newborn and on its future state health, according to the hypothesis of fetal programming.

Significantly higher concentrations of F2-IsoPs have also been found in amniotic fluid in pregnancies with *preterm premature rupture of membranes* (pPROM) compared to pregnancies without pPROM (Longini M, et al. 2007), highlighting the importance of the ROS-induced damage on the amniotic epithelium and the chorio-amniotic collagen. Kwiatkowski S. et al confirmed these results, reporting increased levels of F2-IsoP in amniotic fluid but also in maternal plasma in cases of pPROM (Kwiatkowski S, et al. 2009).

Finally, a significant increase in OS biomarkers and in particular in IsoPs levels was found in the amniotic fluid in case of pregnancies with *Down syndrome fetuses*, with a nine-fold increase in IsoPs concentrations compared to normal fetuses but also higher concentrations than in fetuses with FGR (Perrone S, et al. 2007).

3.3 OS biomarkers in neonatal diseases: clinical-laboratorist correlates

Due to the high susceptibility to oxidative stress of fetus and newborn, especially preterm, we have already stated that the term Free Radicals Disease (FRD) has been coined for a series of typical diseases related to prematurity, in which oxidative damage plays a determining role demonstrable through the assay of OS biomarkers. Elevated levels of TH, AOPP and NPBI in umbilical cord blood have been found to be linked to an increased risk of FRD (Perrone S, et al. 2010).

Elevated plasma TH levels were found in *hypoxic preterm infants*, providing indirect evidence for increased generation of FRs under hypoxic conditions. A correlation between TH and hypoxanthine in the plasma of preterm infants strongly suggests that the deeper the hypoxia, the greater the production

of reactive oxygen metabolites; the degree of hypoxia seems also correlated with AOPP levels, indicating that plasma proteins are affected by FRs damage in preterm hypoxic infants (Buonocore G, et al. 2000; 2002).

Even in *respiratory distress syndrome* (RDS), the most frequent complication of preterms, the role of OS has been demonstrated: MDA, protein carbonyls and 7,8-OHdG were significantly increased, with instead reduced levels of total antioxidant capacity (TAC) in the newborns with RDS compared to healthy newborns (Negi R, et al. 2014). In a recent study, significantly higher plasma levels of carbonylated proteins were confirmed in preterm infants with RDS compared to healthy preterm infants (Ahmed AE, et al. 2017).

In recent decades, emerging data have suggested that OS is involved in lung development and in that of *bronchopulmonary dysplasia* (BPD) in the preterm infant, and that the lung injury process leading to BPD occurs within hours or days of delivery. Neonatal lung damage recognizes multiple etiological factors that act synergistically; a complex interrelation between inflammation and OS represents the physiopathological background that predisposes an immature lung to the development of BPD, with an important contribution due to premature exposure to hyperoxia (Perrone S, et al. 2012; Wang J, Dong W. 2018). Human studies have shown a quantitative increase in oxidative damage to lung proteins and lipids, and a decrease in antioxidant levels in the biological fluids of ventilated preterm infants (Buczynski BW, et al. 2013). In particular, premature infants who subsequently developed BPD and those mechanically ventilated with a high oxygen requirement showed a higher carbonyl content in lung fluid than those who did not develop BPD or who required less oxygen (Gladstone IM Jr , Levine RL. 1994; Varsila E, et al. 1995). Collard et al. demonstrated in the epithelial lining fluid of preterms that developed BPD significantly higher concentrations of MDA than in those that were not oxygen-dependent (Collard KJ, et al. 2004). More recently, in preterm infants who subsequently developed BPD have been found higher urinary levels of 7,8-OHdG than in infants who did not develop BPD, with a positive correlation between these levels on the third day of life and the duration of ventilation (Joung KE, et al. 2011).

Hyperoxia and OS are also involved in the physiopathology of *retinopathy of prematurity* (ROP). The role of OS in this condition is complex and strictly correlated with inflammatory, angiogenic, metabolic and genetic factors. In a previous study, the Authors found no significant differences in OS biomarkers (NPBI, TH, AOPP and carbonyl groups) between premature infants with stage 1-2 ROP and infants without ROP. However, a significant decrease in NPBI levels and an increase in TH were found in the first 3 weeks in both groups, suggesting that all preterms are physiologically prone to OS at birth because the extrauterine environment is richer in oxygen than the intrauterine one (Perrone S, et al. 2009). Furthermore, other Authors found a significant difference in leukocyte and urinary 7,8-OHdG levels and in plasma and urinary MDA levels in patients with ROP compared to those without ROP, making them usable as possible screening tools for the same ROP (Ates O, et al. 2009).

Several studies have suggested a role of OS in the pathogenesis of *necrotizing enterocolitis* (NEC) (Aceti A, et al. 2018; Perrone S, et al. 2014). Aydemir et al. found that preterm with NEC had significantly higher Total Oxidant Status (TOS) and Oxidative Stress Index (OSI) levels than controls without NEC, with the highest levels of TOS and OSI associated with major severity of NEC (Aydemir C, et al. 2011). Furthermore, a strong association was found between the concentration of OS biomarkers in cord blood and the presence of NEC in premature babies; in particular, AOPP, TH and NPBI cord blood levels were significantly higher in infants with NEC than in controls (Perrone S, et al. 2012). Ozdemir et al. also reported a significant increase in intestinal MDA in an animal model (neonatal rats) of NEC (Ozdemir R, et al. 2013). NEC is a multifactorial disease with little known pathogenesis, however OS and inflammation are key elements closely related. Many inflammatory biomarkers have been studied (i.e. cytokines IL-6, IL-8/CXCL8, IL-10 and IL-18), however diagnostic accuracy is not optimal and today no single biomarker has been identified as diagnostic in clinical practice (Gephart SM, et al. 2018).

OS also plays a key role in *post-ischemic kidney damage*. In the first two weeks of life, AOPP and TH seemed to be significantly correlated with alpha-1 microglobulin and N-acetyl-bD-glucosaminidase (a microprotein and a tubular enzyme, respectively, that represent a clinical tool for the evaluation of tubular dysfunction), as expression of renal damage induced by oxidative stress in premature infants (Perrone S, et al. 2007).

Patent ductus arteriosus (PDA) is a possible complication of prematurity, and can be related to kidney damage, NEC, BPD, or IVH. Urinary levels of IsoPs showed significant changes in preterms with PDA before and after treatment with ibuprofen. Indeed, a significant decrease in IsoPs has been demonstrated 12-24 hours after pharmacological closure with ibuprofen, followed by a rebound on the seventh day after treatment, suggesting the potential antioxidant effect of this drug in preterm infants with PDA at high risk of OS (Longini M, et al. 2011). Inayat and coll. partially confirmed these findings in a recent study, finding an increase in isoprostane (8-isoPGF2α) levels after treatment, possibly due to increased oxygenation and ROS levels, although they report lower pre-treatment IsoPs levels in preterm infants who have developed hemodynamically significant PDA (Inayat M, et al. 2015).

Probably the most studied FRD of prematurity is *intraventricular haemorrhage* (IVH), frequently associated with long-term consequences along with the possible subsequent *periventricular leukomalacia* (PVL). Since it is now accepted that FRs contribute to preterm brain damage, early diagnosis of the presence of OS-related damage by a validated panel of biomarkers is required to prevent long-term OS-related sequelae (Panfoli I, et al. . 2018). A significant association between preterm umbilical cord blood TH, AOPP and NPBI levels and subsequent development of all grades of IVH has been demonstrated; this finding suggested that the increase in OS biomarkers is a direct index of an increased production of FRs in the central nervous system as a response to oxidative neuronal damage (Perrone S, et al. 2010). An increase in NPBI was found in the cerebrospinal fluid of preterm infants with PHVD, suggesting an association between IVH and subsequent white matter damage (Savman K, et al. 2001). Furthermore, NPBI appears to be the best early predictor of neurodevelopmental outcome

(Buonocore G, et al. 2003). IsoPs are other reliable biomarkers of brain injury (Tataranno ML, et al. 2015): an interesting recent study showed that in the first month after birth, increases in plasma IsoPs identify preterm infants at risk of respiratory morbidity at term-equivalent age and at risk of worse developmental outcomes at 12 months of corrected age, with poor neurological development largely independent of respiratory morbidity (Matthews MA, et al. 2016).

However, even the brain of the full-term infant is particularly vulnerable to the OS-related damage. The predictive role of a predefined panel of OS biomarkers for the early identification of newborns at high risk of *hypoxic-ischemic encephalopathy* (HIE) and their validation through correlation with MRI results has recently been reported: the presence of an association between biomarkers of oxidative stress measured in the first hours of life and brain damage (successfully evaluated by neuroimaging), underlines the possibility of early identification of newborns at greater risk of brain damage. This finding also underlines the validity of AOPP, as products of OS damage in plasma and therefore as biomarkers of neuronal damage (Negro S, et al. 2018).

CHAPTER 4

OXIDATIVE STRESS AND POTENTIAL PREVENTIVE-THERAPEUTIC STRATEGIES IN THE NEWBORN

4.1 Antioxidants: types, functions and clinical uses in neonatal setting

An antioxidant is any substance capable of eliminating ROS and their derivatives; enzymatic antioxidants and non-enzymatic scavengers can be distinguished, and their functions can be classified according to their mechanisms of action: preventive agents that suppress the formation of new radicals, including enzymes such as SOD, CAT and GPX or metal-binding proteins such as ferritin and ceruloplasmin, and minerals such as selenium (Se), copper (Cu) and zinc (Zn); agents that eliminate radicals by inhibiting the initiation and/or propagation of the FRs production chain, such as glutathione, albumin, vitamins C and E, carotenoids and flavonoids; repair enzymes, that reconstitute cell membranes damaged by oxidative damage and include lipases, proteases, DNA repair enzymes, transferases and methionine sulfoxide reductase; adaptation agents that generate appropriate antioxidant enzymes and transfer them to the essential site of action (Falsaperla R, et al. 2020).

Antioxidants can therefore be classified into several lines of defense. Non-enzymatic agents that are part of the first line of defense are "preventive" antioxidants, and include plasmatic proteins like ceruloplasmin, ferritin, transferrin and albumin: they inhibit the formation of new reactive species by binding transition metal ions (for example iron and copper). The second line of defense against ROS is represented by molecules characterized by the ability to rapidly inactivate radicals and oxidants. The third line of defense consists in the repair mechanisms against the damage caused by ROS and FRs: this form of protection is provided by enzymatic antioxidants, that can repair damaged DNA and proteins, stop the propagation of the lipoperoxy radical chain and repair cell membranes and damaged molecules; food antioxidants such as vitamin E, vitamin C, carotenoids, some minerals (e.g. Zn, Mn, Cu, Se) and polyphenols can influence the activity of endogenous antioxidants, with a synergistic action aimed at maintaining or restoring redox homeostasis (Mirończuk-Chodakowska I, et al. 2018).

4.1.1 Enzymatic antioxidants

Superoxide dismutase (SOD; EC1.15.1.1) is the antioxidant enzyme that catalyzes the dismutation of the highly reactive superoxide anion to O2 and H2O2. There are three forms of SOD in humans, namely cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD and extracellular SOD, of which the first (Cu/Zn-SOD) plays an important role in the first line of antioxidant defense; elevated SOD activities have been correlated with high immune competence. CAT and GPX, on the other hand, play an important role in H2O2 detoxification. *Catalase* (CAT; EC1.11.1.6) reacts very efficiently with H_2O_2 to form molecular water and oxygen, and with hydrogen donors with peroxidase activity, thus protecting cells from the H2O2 generated within them. *Glutathione-peroxidase* (GPX; EC1.11.1.19) catalyzes the neutralization reactions of hydroperoxides and H_2O_2 using GSH glutathione, whose metabolism is one of the more relevant antioxidant defense mechanisms (Georgeson GD, et al. 2002). Delivery and birth constitute a

significant time of oxidative stress exposure, and the duration of gestation and the circumstances of delivery influence the overall oxidative load. The enzymatic activities are measurable, and it has been observed that the antioxidant defense mechanisms of the newborn are profoundly modulated by gestational age and mode of delivery: in particular, the activity of CAT was significantly higher in full-term than in preterm births and, similarly, in infants born from spontaneous vaginal birth compared to those born with a caesarean section; gestational age was also a determining factor in activity levels for SOD and GPX (Georgeson GD, et al. 2002). Specific polymorphisms in the genes coding for these antioxidant enzymes are related to the risk of onset of prematurity/OS-related diseases (Poggi C, et al. 2012). Furthermore, EPTs born from mothers treated with prenatal betamethasone (anti-RDS prophylaxis) have shown a greater activity of SOD, catalase and glutathione-S-transferase in the umbilical cord blood and in the neonatal blood obtained 24 hours after delivery, also with a tendency to increased GPX and glutathione-reductase activities, compared to EPT controls from untreated mothers (Vento M, et al. 2009).

4.1.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants intercept and terminate the chain reactions of free radicals; they can be endogenous or exogenous (dietary origin), and include multiple substances such as vitamins E, A, C, coenzyme Q10 (CoQ10), flavonoids, carotenoids (including lutein), glutathione, plant polyphenols (flavonoids), uric acid, caffeine, allyl sulfides, curcumin, melatonin, bilirubin and polyamines (Ziad M, et al. 2019). They can also be distinguished into intracellular compounds (such as ferritin, CoQ10, glutathione, melatonin) and extracellular compounds (such as transferrin, lactoferrin, albumin, uric acid, unconjugated bilirubin).

Vitamin A is a carotenoid produced in the liver and derived from the breakdown of β-carotene (see below, carotenoids). It can be found as retinol, retinal and retinoic acid in the body, and all these forms are usually bound to proteins in extracellular fluids and within the cells, because are toxic at high concentrations; vitamin A is therefore stored mainly as long-chain fatty esters and as provitamin in the liver, kidneys and adipose tissue (Palace VP, et al. 1999). It can directly bind peroxyl radicals before they trigger the propagation phase of lipid peroxidation (He L, et al. 2017).

CoQ10 can neutralize the oxidative effect of lipoperoxy radicals and regenerate vitamin E (He L, et al. 2017). It is involved in the transport of electrons in the mitochondrial respiratory chain and in the transport of electrons outside the mitochondria, and participates in the redox reactions of dehydrogenases, cytochromes or other non-heme proteins; these properties are demonstrated only for the reduced form of CoQ10, ubiquinol (CoQ10H2), and for the radical ubisemiquinone (CoQ10H), that in turn has antioxidant properties and can react with molecular oxygen and other FRs (Mirończuk-Chodakowska I, et al. 2018). Vitamin C or ascorbic acid is effective in eliminating the superoxide anion radical, hydrogen peroxide, hydroxyl radical, singlet oxygen and reactive nitric oxide.

Vitamin E is a powerful antioxidant that reduces and removes the lipid peroxyl radical, an initiator of lipid peroxidation within the cell membrane. It has 8 isoforms that block lipid peroxidation, donating

phenolic hydrogen to the peroxyl radicals that thus form non-reactive tocopheroxyl radicals and therefore unable to continue the oxidative chain reaction; Vitamin E can be regenerated through vitamin C, with a combined and synergistic effect of their antioxidant potential.

Minerals are a small percentage of antioxidants of food origin, the most important of which are selenium and zinc, enzymatic cofactors and therefore important for maintaining the activity of enzymatic antioxidants. *Zinc* has multiple functions: it is an inhibitor of NOX, that catalyzes the production of the singlet oxygen radical from oxygen using NADPH as an electron donor; it is also a component of SOD1 (the important antioxidant enzyme that converts the singlet oxygen radical into hydrogen peroxide); it induces the production of metallothionein, which is a scavenger of the hydroxyl radical; it also acts as an effective anti-inflammatory agent (He L, et al. 2017).

Carotenoids are colored fat-soluble pigments found in plants, fungi, bacteria and algae and in many foods; there are more than 600 with natural structural variants that are divided into carotenes and xanthophylls: xanthophylls (for example lutein, zeaxanthin) contain oxygen as a functional group, while carotenes (α- and β - carotene and lycopene) contain only a hydrocarbon chain mother without any functional group; they can also be distinguished into provitamin A and non-provitamin A compounds. Only about 40 carotenoids are present in the human diet and about 20 have been identified in human blood and tissues, including β-carotene, α-carotene, lycopene, lutein and cryptoxanthin. Carotenoids are ROS scavengers such as singlet molecular O2 and peroxyl radicals; they can eliminate radicals in three stages: electron transfer (oxidation, reduction: CAR + ROO → CAR + ROO−), hydrogen extraction (CAR + ROO → CAR + ROOH) and hydrogen addition (CAR + ROO → ROOCAR). The presence of conjugated double bonds allows these compounds to accept electrons from reactive species and thus neutralize the FRs. A combination of lipophilic antioxidants (e.g. vitamins E, C and β-carotene) leads to synergistic effects in scavenging reactive nitrogen species (RNS) and inhibiting lipid peroxidation (Milani A, et al. 2017).

Among the *xanthophylls*, zeaxanthin and lutein in particular act as antioxidants protecting photoreceptor cells from the potential damage caused by FRs. *Flavonoids* (flavonols, anthocyanins, isoflavonoids, flavanones and flavones) are a large class of polyphenols found in plants. They have an antioxidant activity that depends on the structural arrangement of the functional groups, as both the configuration and the total number of phenolic hydroxyl groups substantially influence their mechanism of action; they have excellent chelating properties for iron and other metals in their structure, that is probably their main biological function (He L, et al. 2017; López JG. 2019). Today, however, new hypotheses have been postulated on possible mechanisms of action, including the influence of the interaction of polyphenols with the gut microbiota and the possibility that flavonoids or their metabolites could modify gene expression or act as potential modulators of intracellular signaling cascades (González-Paramás AM, et al. 2019).

Among the endogenous antioxidants we include *metalloproteins* or *metal-binding proteins* (MBPs), i.e. extra- and intra-cellular proteins such as albumin (ALB), ceruloplasmin (CP), metallothioneins (MT),

ferritin (FER), myoglobin (MB), transferrin (TF) and lactoferrin (LTF). MBPs are the main contributors to the antioxidant capacity of plasma, and their antioxidant properties imply their ability to bind transition metal ions, such as Cu^{2+} and Fe^{2+}, that can be extremely pro-oxidizing as they can react with H2O2 and catalyze the formation of ROS in the Fenton reaction; some of these proteins can also act as real scavengers of reactive species. In particular, transferrin, ferritin and lactoferrin are active redox iron (Fe^{2+}) chelators, effective inhibitors of FRs in the Fenton reaction; ceruloplasmin acts as an inhibitor of reactive species by binding free copper Cu^{2+} and iron ions Fe^{2+} or as a chain-breaking antioxidant. Albumin is a multifunctional antioxidant protein, which binds the redox metals Cu^{2+} and Fe^{2+} and can also act as a true scavenger by reacting with hydroxyl radicals. Myoglobin is an effective NO scavenger. Metallothionein is able to bind active redox metal ions such as Cu^{2+} and stable redox metal ions such as Zn, protecting cells from toxic metals, and also acts as a scavenger of reactive species (Mirończuk-Chodakowska I, et al. 2017).

Glutathione (GSH) is a low molecular weight compound composed of three amino acids: glycine, cysteine and glutamic acid (l-γ-glutamyl-l-cysteinyl-glycine). Under physiological conditions it is synthesized in many different tissues, mainly in hepatocytes; in the human body it is present in different redox forms, among which the most important are reduced glutathione (GSH) and oxidized glutathione (GSSG): under normal conditions, the predominant cellular form of glutathione is its reduced form (GSH) in a ratio of 100: 1. Glutathione has multiple functions: it participates in the detoxification processes of electrophilic compounds (xenobiotics) and in the metabolism of Pg and leukotrienes; it is involved in the transport of amino acids and in the absorption of micronutrients from the intestine, mainly iron and selenium. However, the predominant role of GSH is undoubtedly the antioxidant one, contributing to several lines of defense against ROS: not only is it a free radical scavenger, but it is also involved in the repair processes of damaged cells. The antioxidant properties of GSH depend on two characteristics of its molecule, namely the presence of a special pseudo-peptide bond between the amino group of cysteine and the alpha-carboxylic group, that provides excellent protection against aminopeptidases, and the expression of the thiol group (-SH) deriving from the cysteine residue, which is among the most reactive chemical species: the most important functions of the -SH groups in biological systems include the complexation of metal ions, participation in oxidation reactions (whose final product is sulphonic acid) and the formation of thiol radicals and disulfides. As an antioxidant, GSH reduces ROS during enzymatic and non-enzymatic reactions; regenerates other small oxidized antioxidant molecules, eg vitamin C and vitamin E; is involved in the repair of protein molecules, nucleic acids and lipids damaged in the processes of peroxidation and in the maintenance of sulfhydryl protein groups in the reduced state (Mirończuk-Chodakowska I, et al. 2018). An increase in GSH synthesis is part of the adaptive response to OS, which involves three pathways: the first is an increase in the ability to reduce GSSG to GSH through the action of GSSG-reductase; the second is an increase in the synthesis of GSH de novo through the induction of glutamatocysteine ligase (GCL); the third is an increase in the

γ-glutamyl transpeptidase (GGT) enzyme which is found on the outer surface of cells and catalyzes the transfer of the γ-glutamyl fraction of GSH to amino acids (Forman HJ. 2016).

N-acetylcysteine (NAC) is the N-acetyl derivative of the natural amino acid l-cysteine (cys); it is a powerful antioxidant compound whose in vivo activity can be traced back to at least three different mechanisms: a direct antioxidant effect towards some oxidizing species; an indirect effect as a result of the ability of NAC to act as a precursor of Cys, a constituent and rate-limiting element in the synthesis of glutathione; a disruption effect on disulfides and the ability to restore thiol group pools, that in turn regulate the redox state (Aldini G, et al. 2018).

Returning to the endogenous metabolites, *uric acid* is one of the low molecular weight organic compounds, generated during the metabolism of purines; it is a hydrophilic antioxidant, responsible for two thirds of the total oxygen elimination activity in serum, being a scavenger of various ROS such as peroxynitrite, hydroxyl radical, singlet oxygen and lipid peroxides (Mirończuk-Chodakowska I, et al. 2018): it can prevent peroxynitrite-induced protein nitrosylation, lipid and protein peroxidation and inactivation of tetrahydrobiopterin, resulting in the elimination of FRs and chelation of transition metal ions (He L, et al. 2017).

Many studies have emphasized the antioxidant role of *bilirubin*, in particular unconjugated bilirubin is able to eliminate singlet oxygen with high efficiency, to react with superoxide anions and peroxyl radicals and to serve as a reducing substrate for peroxidases in the presence of peroxide of hydrogen or organic hydroperoxides; however, although the antioxidant effect of bilirubin as a ROS scavenger is well documented in vitro and in animal studies, its role in vivo -especially in neonates- is still debated (Dani C, et al. 2019).

Melatonin (N-acetyl-5-methoxytryptamine; MEL) is an endogenous molecule, ubiquitous in nature; it is a neurohormone secreted by the pineal gland, synthesized by the neurotransmitter serotonin and recognized as a "ubiquitously distributed and functionally different molecule". More commonly known as the "sleep hormone", it also has antioxidant, anti-inflammatory, anti-apoptotic properties and many other crucial functions such as the regulation of the circadian rhythm and a powerful analgesic action (D'Angelo G, et al. 2020; Tarocco A, et al. 2019). Melatonin acts through pleiotropic mechanisms of action: some are not mediated by cell receptors and involve the direct interaction of MEL with other molecules (such as its antioxidant action: it is in fact one of the most powerful natural antioxidants, being able to directly chelate ROS and RNS or mobilize and stimulate the intracellular antioxidant enzymatic system); other mechanisms are instead mediated by specific cellular receptors, as occurs for any other hormone (Amaral FGD, Cipolla-Neto J. 2018). MEL is both an adept direct scavenger of free radicals (and so are its metabolites), and an activator of scavenging mechanisms such as stimulation of transcription and antioxidant enzyme activity or transition metal binding with subsequent inhibition of the formation of the hydroxyl radical. MEL protects lipids, proteins and DNA from oxidative damage, being highly concentrated in the mitochondria: its antioxidant properties are crucial for mitochondrial functions, the site where FRs are naturally originated from cellular respiration; at the mitochondrial level

MEL plays a role in the regulation of the activities of the respiratory chain complexes I and IV and in the protection of mitochondrial DNA from mutations and deletions (Cipolla-Neto J, Amaral FGD. 2018). Melatonin's direct antioxidant and FRs scavenging properties are primarily due to its electron-rich aromatic indole ring, making it a powerful electron donor capable of significantly reducing oxidative stress. In addition to this direct action, melatonin can further activate the melatonin receptors MT1 and MT2, up-regulating the antioxidant defensive systems through the increase in the expression or activity of antioxidant enzymes such as SOD and GPX (Tarocco A, et al . 2019). Pineal MEL is not stored, being readily released into the blood (where it binds to albumin) and CSF, reaching different areas of the CNS and all peripheral organs; its half-life in the blood is ~ 40 minutes. Maternal MEL is the only source of this hormone for both the fetus (via placental circulation) and the infant (via breastfeeding), as their pineal glands do not produce melatonin until after birth, moreover with an irregular discharge in the first months of life. Pineal melatonin has been demonstrated in full-term infants at the age of 3-4 months, peaking in prepubertal children, and decreasing after puberty to young adult levels (Cipolla-Neto J, Amaral FGD. 2018).

4.1.3 Roles and clinical uses of antioxidants in the newborn

As antioxidant systems mature rapidly during the third trimester of pregnancy, in preterm infants they are immature and deficient. Many antioxidant drugs have therefore been used in clinical and experimental approaches to reduce OS in oxygen-related neonatal diseases, but the results are still uncertain for most of them (Perez M, et al. 2019; Buonocore G, Groenendaal F. 2007).

Antioxidants that have been clinically targeted to prevent complications of prematurity include recombinant enzymes such as Cu-ZnSOD, MnSOD and ecSOD; the GSH; vitamins such as A or E; trace elements which are enzymatic cofactors, such as selenium or L-arginine. Their development as clinical therapies has however been limited by biochemical and physiological factors: biochemical obstacles are represented by the half-life of the compounds, the poor cellular penetrance and the difficulty in targeting the intracellular organelles; furthermore, there is a lack of physiological knowledge about ideal antioxidant levels, the appropriate enzyme activity for gestational age, the relationship between cofactor levels and enzyme activity in the preterm infant, as well as the appropriate disease endpoints to assess therapeutic effects (Perez M, et al. 2019). Through some randomized clinical trials, although dated, it has been observed that neither vitamin A nor vitamin E effectively reduce the risk of BPD or death in ELBW infants; Vitamin E supplementation in preterm infants appears to reduce the risk of IVH and severe ROP, but also to increase the risk of sepsis and therefore evidence does not support routine use; Vitamin C supplementation in very preterm infants did not show significant benefits nor harmful effects; treatment with enzymatic antioxidants (ROS inhibitors and scavengers, i.e. SOD, GPX and CAT) has shown several limitations including a prolonged time to penetrate the blood brain barrier, a narrow therapeutic dosage range, a protective efficacy only if administered much earlier than the insult; therapeutic intervention with the infusion of N-acetylcysteine (precursor of glutathione and, in itself, a free radical scavenger) did not show beneficial effects (Buonocore G, Groenendaal F. 2007).

Regarding the clinical outcomes investigated, *BPD* is probably the most studied. Exogenous antioxidants such as vitamins A, E and recombinant human SOD (rhSOD) have been administered in an attempt to prevent BPD. Although a Cochrane meta-analysis suggested that vitamin A supplementation reduces BPD, neurodevelopmental outcomes at 18-22 months of corrected GA were not significantly different in treated groups compared to controls (Tyson JE, et al. 1999). Randomized controlled trials of vitamin E supplementation also failed to show a reduction in the incidence of BPD, and even studies examining trace elements as active cofactors in ELBW infants showed that lower trace element concentrations did not affect substantially the antioxidant enzymes concentration nor the development of BPD (Lee JW, Davis JM. 2011; Perez M, et al. 2019). As regards enzyme replacement, one of the most relevant clinical studies for the prevention of long-term pulmonary complications involved the intratracheal administration of rhSOD: although rhSOD administration has not decreased early death or BPD incidence, at one year survivors had a reduced lung disease burden quantified by the number of emergency department visits, drug use, and hospital readmissions for lung causes (Davis JM, et al. 2003). Attempts to correct GSH deficiency in premature infants with NAC did not affect the incidence of BPD or lung function in infancy, and similarly, despite promising animal studies, clincial studies using inhaled NO early in preterm infants did not show a useful result in preventing BPD (Perez M, et al. 2019). In an in vivo study, it was shown that hyperoxia-induced increases in nitrite/nitrate levels, myeloperoxidase and MDA can be prevented by melatonin; furthermore, other authors have shown - in animal models - that nocturnal administration of melatonin hinders interstitial fibrosis and the reduction of the total number of alveoli associated with BPD (D'Angelo G, et al. 2020), and this evidences would merit further investigation in models humans.

The effects of the use of antioxidants (especially NAC) on the development of *ROP* have also been investigated, however without definitive therapeutic evidence (Lee JW, Davis JM.2011). Several studies regarding vitamin E supplementation in preterm infants to prevent or limit ROP have yielded mixed results, and its use has now been largely abandoned due to increased morbidity risks without conclusive evidence for prevention against severe ROP. Lutein and zeaxanthin have been investigated for their antioxidant action in the eye; in particular, the antioxidant and anti-inflammatory properties of lutein are related to its unique chemical structure. Studies have shown that lutein can directly protect against H_2O_2-induced OS at the ocular level, however supplementation with lutein has been shown to be ineffective in preventing ROP in premature infants (Aranda JV, et al. 2019). The administration of lutein in the first hours of life of full-term infants was associated with a significant decreases in the oxidative stress biomarkers (TH, AOPP) levels at 48 hours, thus confirming its antioxidant role in neonatal age, so further clinical studies seem necessary (probably investigating different dosages of lutein) to evaluate the therapeutic effects of this substance on the free radicals-related diseases, especially in the preterm (Perrone S, et al. 2014). Vitamin A supplementation in extremely preterm infants has recently been shown to be associated with a reduced incidence of type 1 retinopathy of prematurity (ROP1) (Sun H, et al. 2020).

There is a great deal of literature data regarding the potential neuroprotective effects of antioxidants; the HIE of the term newborn is particularly investigated, however several antioxidant agents are also being studied for the prevention of *brain damage* in the preterm infant (PVL). Melatonin is the antioxidant with the best known neuroprotective effects, demonstrated in animal models (Lee JY, et al. 2019) and in term infants (McNally MA, et al. 2019); in the preterm infant, however, further investigations are necessary. A multicentre randomized controlled study aimed at evaluating the neuroprotective effect of intravenous melatonin in preterm infants <31 weeks GA found no differences in the fractional anisotropy of WM at neuroimaging (Merchant N, et al. 2014). The "Protect Me Trial", that aims to evaluate the effect of maternal melatonin supplementation in early-onset FGR pregnancies on the neurodevelopmental outcomes of preterm births at 2 years of age (Palmer KR, et al . 2019), and an Italian clinical trial aimed at investigating the early and long-term neuroprotective effect of oral MEL supplementation in infants <30 weeks GA (Garofoli F, et al. 2021), are actually underway. Among other antioxidant agents, erythropoietin (EPO) has been shown in clinical studies to be beneficial in reducing neonatal brain damage in term and preterm neonates, with neuroprotective and neuroregenerative effects likely related to its anti-inflammatory, anti-excitotoxic, antioxidant and anti-apoptotic effects on neurons and oligodendrocytes and to the regenerative effects of oligodendrogenesis, neurogenesis and angiogenesis; other studies have shown a role for magnesium in the prevention of preterm neonatal brain injury (McNally MA, Soul JS.2019; Singhi S, Johnston M.2019). Long-term vitamin E (alpha-tocopherol) supplementation appears to improve mental development, particularly performance IQ, in ELBW school-age children (Kitajima H, et al. 2015).

Regarding the prevention of *NEC*, a systematic review about L-arginine supplementation showed a protective effect, however the studies considered in the review included only 235 infants, thus limiting the confidence in adopting this therapy as a clinical practice (Mitchell K, et al. 2014). The use of other antioxidants (eg quercetin) in the NEC is still limited to animal and experimental models (Wang K, et al. 2019; Lee JW, Davis JM.2011).

Recently, several authors have reviewed and summarized the most recent and relevant preclinical trials and randomized clinical trials on the use of antioxidants in preterm infants (Perez M, et al. 2019; Falsaperla R, et al. 2020), but in conclusion, there is still a lack of strong evidence in humans to encourage the use of antioxidant therapies as a standard of care, and further work is needed to understand the antioxidant system in preterm infants, to determine their ideal enzyme activity, and to understand how histopathological improvements observed in animal models correlate with physiological outcomes in human infants (Perez M, et al. 2019).

In the following paragraph a clinical trial, result of a multicentre collaboration, carried out on preterm infants and aimed at evaluating the antioxidant efficacy of melatonin is illustrated (see the Annex 9 below for the published article).

4.2 "*Antioxidant effect of melatonin in preterm newborns*" (Published on Oxidative Medicine and Cellular Longevity 2021; 2021:6308255).

4.2.1 Introduction

Preterm infants are at risk for neonatal disorders related to immaturity. A common factor in the pathogenesis of such diseases is the free radicals mediated tissue injury derived from oxidative stress (OS) (Perrone S, et al. 2018). The endogenous indoleamine melatonin, synthesized from the neurotransmitter serotonin, is a powerful broad-spectrum antioxidant and readily available scavenger of free radicals. Foetal melatonin has a maternal origin, and after birth, the full-term neonates have an irregular melatonin secretion for 3-5 months, leading to a transient melatonin deficiency in neonatal period and in the first months of life. Prematurity delays the maturation of the neurological network that controls melatonin secretion, leading to poor secretion for an even longer period. Furthermore, the onset of pineal melatonin secretion seems to be even more delayed in case of neurological damage, and this event, together with other predisposing conditions, makes the preterm even more susceptible to the free radicals-mediated damage (Muñoz-Hoyos A, et al. 2007; Kennaway DJ, et al. 1996; Biran V, et al. 2019). Therefore, exogenous melatonin administration appears a promising strategy in the treatment of neonatal morbidities in which OS has a leading role. Moreover, as it shows neuroprotective properties, it was present as a joint therapy in addition to hypothermia after hypoxic-ischemic encephalopathy (Balduini W,et al.2012; El Farargy MS, Soliman NA.2019; Cardinali DP.2019; Hobson A,et al.2013). Several studies tested the efficacy of melatonin to counteract oxidative damage in diseases of newborns such as chronic lung disease, perinatal brain injury, necrotizing enterocolitis, retinopathy of prematurity and sepsis, giving promising results (Xu Y, et al. 2018; Zhang WX, et al. 2019; Tarocco A, et al. 2019). In these studies, the dosages of melatonin varied over a wide range, ranging from 0.1 to 100 mg/Kg. This is an evidence that pharmacokinetic profile of melatonin is better known in adults than newborns (Andersen LP, et al.2016). Indeed, just few studies investigated pharmacokinetic characteristics of melatonin in preterm and asphyxiated neonates. Merchant et al. observed and described a decreases volume of distribution and prolonged half-life and clearance of the melatonin in preterm infants with respect to adults and older children. Melatonin was administering intravenously at the dosage of 0.1 mg/kg for two hours (Merchant NM, et al. 2013). Carloni et al. investigated the melatonin pharmacokinetic at comparable doses after intragastric administration in human preterm infants. The main result of the study was that a single intragastric bolus of 0.5 mg/Kg of melatonin resulted in higher serum melatonin level than adults suggesting the possibility to get and keep therapeutic concentrations with this (Carloni S, et al. 2017). Finally, Balduini et al. demonstrated that a safe and potentially effective dose of melatonin for infants with hypoxic ischemic encephalopathy (HIE) undergoing hypothermia should not exceed 5 mg/Kg, depending on the route of administration (Balduini W, et al. 2019). However, no data are available on the therapeutic efficacy of these specific doses. The aim of this study was to evaluate melatonin concentrations and biomarkers of oxidative stress in preterm infants after early administration of melatonin.

4.2.2 Materials and Methods

This prospective randomized double bind placebo controlled pilot study was conducted at the Neonatology Unit of the Polyclinic in Messina from January 2019 to September 2020. The study was approved by local Ethical Committee (protocol number 42/2018). Written informed consent was obtained from parents. Inclusion criteria were gestational age <37 weeks and normal liver and kidney function tests. Exclusion criteria are all out-born baby, babies with severe congenital malformations, sepsis, inborn errors of metabolism, suffering from perinatal hypoxia or born from mothers with mental disorders, to eliminate conditions that could affect MEL production. Additional exclusion criteria were as follows: withdraw informed consent, insufficient blood sample, and hemolysis of blood sample.

The MEL group received an oral dose of 0.5 mg/Kg of melatonin, once a day in the morning, in the first week of life; the placebo group received 0.5 mL of 5% glucose solution. Newborns received melatonin (Pisolino® Gocce, Pediatrica, Italy) by a nasogastric tube. Pisolino® Gocce contains fructose, purified water, potassium sorbate, sodium benzoate, flavorings, and xanthan gum. The product is present in the register of food supplements of the Ministry of Health website (http://www.ministerosalute.it/alimenti/dietetica) and classified with the following code: 62853.

This product is subject to the European Directive on foods according to the DL n. 169 of 21/05/2004 and not to the European Directive on medicines 2001/20/EC implemented at Italian level with D.L. n. 211 of 06/24/2003. Melatonin administration has a good safety profile with no known adverse effects (Andersen LP, Gögenur I, et al. 2016)

Plasma concentrations of non-protein-bound iron (NPBI) (micromol/L), Advanced Oxidation Protein Products (AOPP) (micromol/dL) and F2-isoprostanes (F2-Isopr) (pg/mL) were determined at 24 and at 48 hours after administration of melatonin. The primary endpoint was to evaluate MEL concentration in MEL group and placebo group. The secondary endpoint was to evaluate biomarkers of OS, such as AOPP, NPBI, F2-isopr in MEL and placebo groups. Further, the occurrence of intraventricular haemorrhage (IVH), necrotizing enterocolitis (NEC), retinopathy of prematurity (ROP) and bronchopulmonary dysplasia (BPD) in all enrolled preterm newborns was analysed.

4.2.3 Procedures

Blood samples (0.5 ml) were collected, by vein puncture, from each newborns at 24 and 48 hours after administration of MEL. The samples were immediately centrifuged (RTM 1500, T 4°C, 10 min) to remove cells and obtain the supernatant, which was then separated into two different microtest tubes, one of which contained BHT (butylated hydroxytoluene), and stored at −80°C. The obtained samples were subsequently analysed to measure melatonin and OS biomarker (AOPP, F2-IsoPs, and NPBI) concentrations. Plasma melatonin concentrations were measured by high-performance liquid chromatography and mass spectrometry (MS/MS) (Agilent Technologies 1200 series system and an ABSciex API 4000 triple-quadrupole mass spectrometer) according to the method of Wang et al (Wang AQ, et al. 2011). Markers of protein and lipid peroxidation were measured by AOPP and F2-IsoPs. Spectrophotometry, Tandem Mass Spectrometer coupled with HPLC and HPLC-DAD system were

used to analyse AOPP, F2-IsoPs, and NPBI (Witko-Sarsat et al.1996; Casetta B, et al. 2012; Paffetti P, et al. 2006). AOPP were measured using spectrophotometry on a microplate reader. The instruments were calibrated with chloramine-T solutions that absorb at 340 nm in the presence of potassium iodide (Witko-Sarsat et al.1996). The LC-MS/MS method of Casetta et al. (Casetta B, et al. 2012) was followed for determination of F2-IsoPs. The methods were centered around an API 4000 Tandem Mass Spectrometer coupled with HPLC Agilent 1200 series, which includes a binary pump. A thermostated well-plate autosampler and a column over. Chromatography separation was carried out at a temperature of 30°C by a mixture of an aqueous solution of acetic acid (Eluent 1) and acetonitrile (Eluent 2). For measurements, the tandem mass spectrometer ren in multiple reaction monitoring with the electroscopy source operating in negative ion mode and by exploiting the transitions m/z 353.3 >193.2 for F2 IsoPs and 357.3 > 197.2 for the internal standard d4-8 iso $PGF_{2a.}$ The method of Paffetti et al. (Paffetti P, et al. 2006) was followed for NPBI measurement with HPLC-DAD system (Agilent 1100 series). The method is based on preferential chelation of NPBI by a large excess of the low-affinity ligand disodium nitrilotriacetic acid. To separate NPBI, a two-step filtration procedure was used: (1) filtration through a 100-kDa Vecta-Spin Micro-Whatman ultracentrifuge filter; (2) filtration through a 20-kDa Vecta-Spin Micro-Whatman ultracentrifuge filter at 13.660 x g and 4°C. The filtrate was injected directly into an isocratic reverse-phase liquid chromatography system using precolumn derivatization with the high-affinity iron ligand DHP, which forms a coloured complex with Fe3+ that absorbs at 450 nm. The analytic system detected iron as a ferric nitrate standard down to a concentration of 0.01 mM.

4.2.4 Statistical analysis

A computer-generated-randomization schedule was used to define supplemented group (MEL group) or control (Placebo group). Due to lacking data on oral melatonin supplementation in preterm newborns, sample size was calculated by G*Power 3.9.1.2 for windows (Faul F, et al. 2007), estimating that between the 2 groups there was a large difference in the concentration of melatonin. Setting: effect size: 0.8, alfa error: 0.5 and power: 0.80, the minimum sample size required was 28. Statistical analysis was performed by SPSS version 25.0 for Windows (IBM, Armonk, NY, USA). Normal distribution of data was evaluated by Kolmogorov-Smirnov test. Data with non-normal distribution and categorical data were evaluated by Mann-Whitney U test and chi-square test, respectively. A p value <0.05 was considered statistically significant.

4.2.5 Results

The flow diagram of the study population from assessment for eligibility to analysis was reported in figure 5 (corresponding to Figure 1 of the article attached below as Annex 10). Out of the 36 consecutively enrolled preterm newborns, 21 received melatonin (MEL group) and 15 received placebo (Placebo group). Table 1 reports baseline characteristics of the enrolled population. Melatonin concentrations were significantly higher in MEL group at 24 and 48 hours (Table 2). In the placebo group, male showed significantly higher concentrations of melatonin than female at 24 hours of life (58.1 $\pm$ 55.4 *vs* 2.8 $\pm$ 3.5; p =0.001); in the MEL group female showed significantly higher

concentrations of melatonin concentration than male at 48 hours of life (302296.3 ± 372402.9 *vs* 22781.0 ± 35155.7; p =0.03). No statistical difference between groups were found in AOPP and NPBI at 24 and 48 hours, also F2-Isopr was not different at 24 hours (Table 2). At 48 hours the mean plasma concentrations of F2-Isopr were significantly lower in the MEL group than in the placebo group (36.48 ± 33.85 *vs* 89.97 ± 52.01 pg/ml, p<0.05; Table 2, figure 6 corresponding to Figure 2 of the published article). No differences between male and female in OS biomarkers were observed.

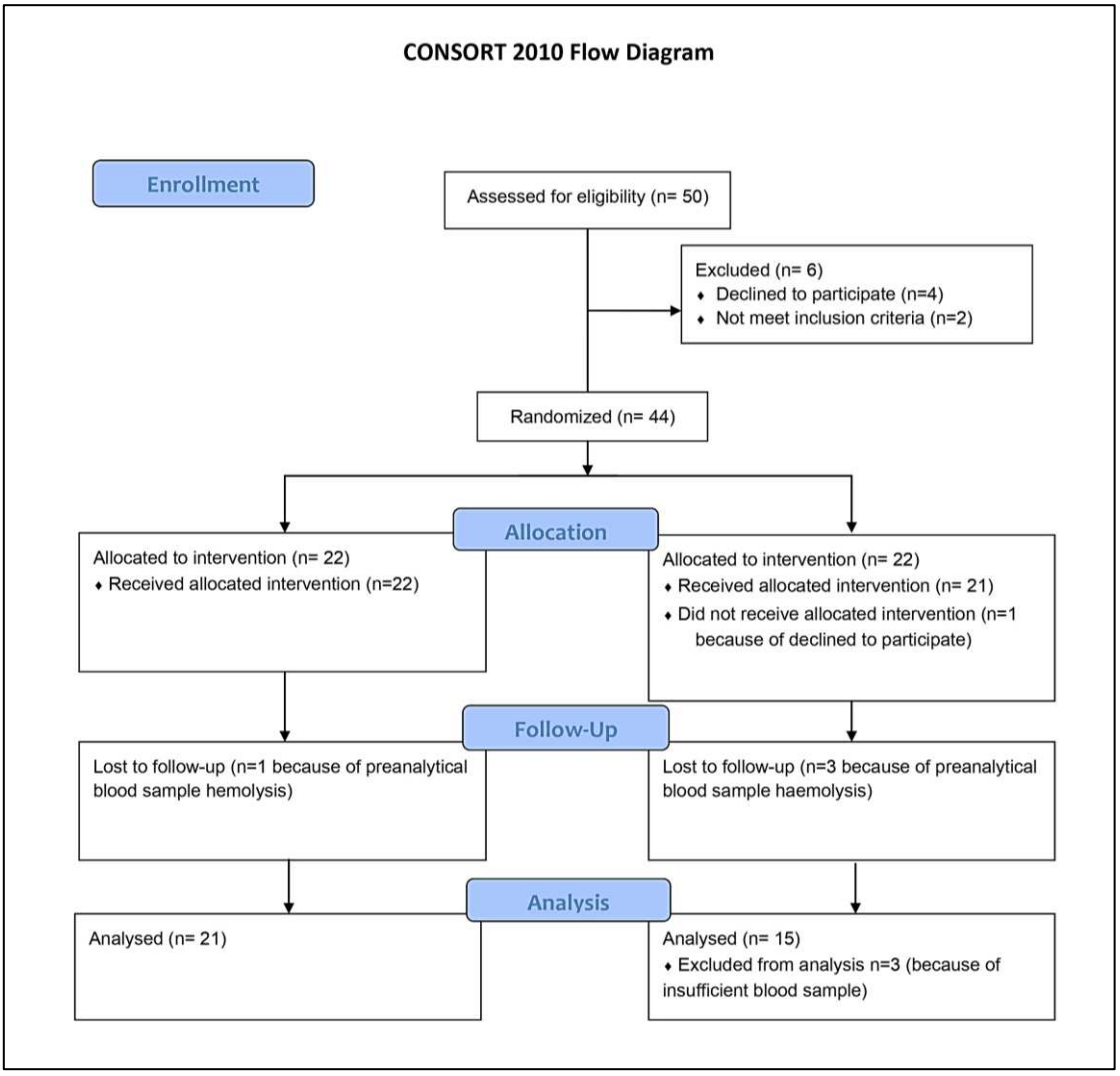

Figure 5: Consort Diagram 2010

Table 1: Clinical characteristics of enrolled population

	MEL group (n = 21)	**Placebo group (n= 15)**	**P_value**
Gestational Age (wks)	32,26 ± 3,66	33,53 ± 2,88	NS
Birth weight (g)	1706,47 ± 637,91	1987,5 ± 513,13	NS
Gender (%)	F=14 (66)	F=8 (53)	NS
Spontaneous delivery (%)	5 (24)	2 (13)	NS
Caesarean Section (%)	16 (76)	13 (87)	NS
NEC (%)	1 (4,7)	0	NS
BPD (%)	1 (4,7)	0	NS
IVH (all grade), (%)	4 (19)	3 (20)	NS

F: female; NEC: necrotizing enterocolitis; BPD: Bronchopulmonary Dysplasia; IVH: intraventricular haemorrhage; ROP: retinopathy of premature; NS= non-significant p>0,05

Table 2: Melatonin, AOPP, NPBI and F2-Isopr levels in test and control groups at 24 and at 48 hours of life

	24 hours			48 hours		
	Placebo Group	**MEL Group**	**p_value**	**Placebo Group**	**MEL Group**	**p_value**
	Mean ± SD [Median (25º-75º)]			**Mean ± SD [Median (25º-75º)]**		
Melatonin (pg/ml)	28.57 ± 46.24 [10(1-43)]	52759.30 ± 63529.09 [18309(8886-100831)]	**<0,001***	38.50 ± 44.01 [17(4-121)]	279397.6 ± 516344.2 [37349 (10108-274844)]	**<0,001***
NPBI (micromol/L)	2.40 ± 3.46 [0.7(0.2-3.3)]	3.97 ± 3.13 [4(1-6)]	0.113	2.99 ± 3.56 [0.8(0.1-6)]	2.23 ± 2.37 [1(0.6-5)]	0.525
AOPP (micromol/dL)	44.66 ± 26.54 [36(28-45)]	36.07 ± 16.03 [32(24-42)]	0.297	54.96 ± 24.33 [53(33-75)]	51.66 ± 18.11 [47(38-60)]	0.715
F2-Isoprostanes (pg/mL)	82.47 ± 51.30 [80(31-121)]	75.05 ± 87.75 [46(20-93)]	0.168	89.97± 52.01 [80(62-127)]	36.48 ± 33.85 [24(10-68)]	**<0.008***

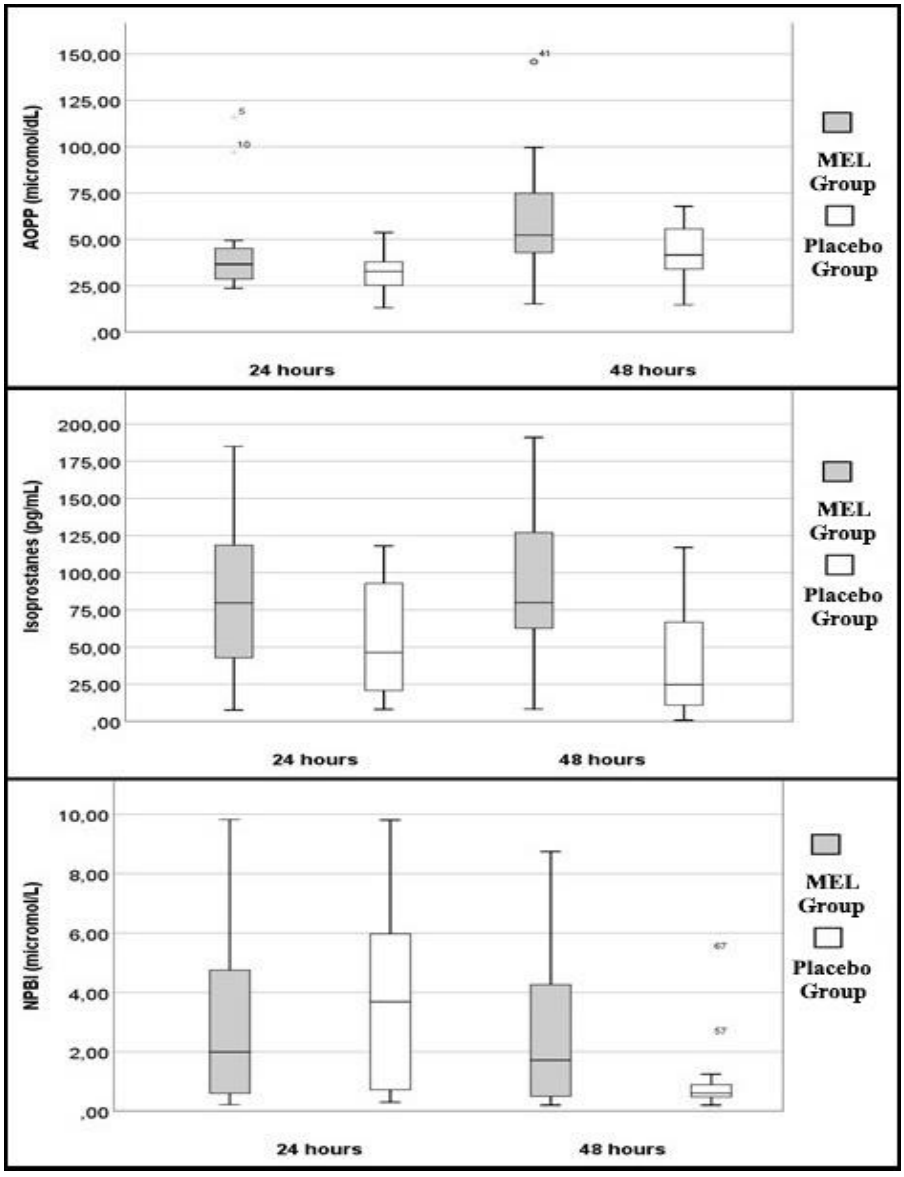

Figure 6: Isoprostanes, NPBI e AOPP concentrations in placebo and MEL groups at 24 and 48 hours after melatonin adminitration

4.2.6 Discussion

The inability to counteract the harmful effects of free radicals is matter of concern for all newborns, especially if preterm. The transition from intrauterine to extrauterine environment is characterized by a huge of oxygen availability (Tarocco A, et al. 2019; Perez M, et al. 2019). This new hyperoxic condition increases the generation of various reactive oxygen species (ROS) such as hydrogen peroxide, singlet oxygen and hydroxyl radicals that may attack macromolecules and cellular components. Moreover, ROS, as a secondary messenger, may trigger signalling pathways and induce stress-response genes or proteins (Perez M, et al. 2019; Tipple TE, Ambalavanan N. 2019). A significant increase in total hydroperoxides and AOPP levels from birth to 7 days of life has been reported in preterm newborns, indicating that damage caused from free radicals also occurs in non-hypoxic babies with normal clinical course (Buonocore G, et al.2002). Experimental studies in animal model of hypoxic-ischemic brain damage report the effectiveness of antioxidants drugs to prevent or reduce ROS injury. Melatonin has been demonstrated to be able to block OS and inflammation pathways (Hardeland R.2018; Wang Z, et al.2018). In the first days of life numerous factors could be responsible for an overproduction of free radicals, such as hypoxia, hyperoxia, acidosis, infections, transfusions, drug exposure, and pain (Di fiore JM, Vento M. 2019). Newborns are therefore peculiarly at high risk for OS-induced damage (Peña-Bautista C, et al. 2019). Therefore, there is compelling evidence that supplementation with antioxidant compounds may be effective in combating OS. Melatonin has not only free radical scavenging and antioxidant properties but also anti-inflammatory, anti-apoptotic and analgesic actions. Indeed, melatonin seems to modulate both pro- and anti-inflammatory cytokines in various pathophysiological situations wherein the balance between them determines the clinical outcome and to inhibit the expression of cyclooxygenase and inducible nitric oxide synthase, the nitric oxide production induced by lipopolysaccharide and the inflammasome activation (Tarocco A, et al. 2019). This fact is of clinical importance if we consider that inflammation is strictly related to OS in the pathogenesis of many diseases that affect preterm newborns (Perrone S, et al. 2018). Previous reports have suggested that preterm infants do not secrete melatonin until 52 weeks post conception (Kennaway DJ, et al. 1992). In our study we were able to measure the melatonin concentration in plasma of preterm infants who received placebo. All subjects received maternal or human donor milk which was a potential source of exogenous melatonin, being present in human milk (Illnerová H, et al. 1993). Melatonin concentrations were found significantly higher in male than female in placebo group at 24 hours of life and in female than male in MEL group at 48 hours of life. To our knowledge, no data on melatonin differences between male and female have been reported. Immature hepatic metabolism and poor renal excretion may be responsible for a wider range of melatonin concentrations in treated preterm babies. Whatever the reason for the observed gender differences, the data should be checked in a large population due to the variability of melatonin concentrations in preterm newborn.

A protective effect of melatonin on lipoperoxidation was observed when orally administered in preterm newborns in the first days of life. Significantly lower levels of F2-IsoPr were found in the MEL than the

placebo group at 48 hours of life. This result is particularly important since early measurement of F2-IsoPr has been recently described to discriminate patients showing abnormal white matter injury score at term of corrected gestational age with a cut off value 31.8 pg/ml (Coviello C, et al. 2021). Moreover, high levels of urinary F2-IsoPr were found in second days of life in newborns at high risk of developing a hemodynamically significant patent ductus arteriosus (Coviello C, et al. 2020). Increased levels of F2-IsoPr have been also reported in preterm newborns affected by bronchopulmonary dysplasia or periventricular leukomalacia (Ahola T,et al.2004). It was demonstrated that F2-IsoPr provokes pre-oligodendrocytes death by oncosis, depending on inadequate antioxidant defences (Brault S, et al. 2004). White matter injury, bronchopulmonary dysplasia, periventricular leukomalacia, and patent ductus arteriosus represent some of the peculiar diseases of prematurity, now grouped and called 'free radical diseases of prematurity' because of the common pathways in pathogenesis (Perrone S, et al. 2018). The results of the present pilot prospective study show that few doses of melatonin decrease lipid peroxidation in preterm supplemented newborns. Thus, melatonin appears to reduce the risk of oxidative damage, protecting vulnerable organs and tissues in preterm newborns. F2-IsoPr are the *in vivo* result of free radical-induced injury by peroxidation of lipids in cell membranes. They are stable compounds generated by the action of cyclooxygenase on long-chain unsaturated fatty acids. The mechanism involved in their formation implies that free radicals cause hydrogen abstraction from arachidonic acid and addition of molecular oxygen to form a peroxyl radical. F2-IsoPr are terminal oxidation products with no further oxidant properties, therefore representing reliable markers of OS in newborns (Longini M, et al. 2017). AOPP are the terminal products of the protein exposure to free radicals without oxidant properties and they represent a marker of the degree of protein damage in oxidative stress conditions. We previously reported an increase of AOPP levels from birth to seventh day of life in preterm newborns (Buonocore G, et al.2002); in this paper we observed a lower relative increment of AOPP level in treated newborns than controls. NPBI is a low-molecular-mass iron form, free from binding to plasma proteins. Iron toxicity derives from the production of hydroxyl radicals through the Fenton reaction. Thus, NPBI is a marker of potential OS because it indicates increased susceptibility to oxidative damage especially in vivo studies (Longini M, et al. 2017). We previously found an association between NPBI and lipid oxidation in vitro (Signorini C, et al. 2008). In this study no significant effect on NPBI and AOPP was observed at 24 and 48 hours from MEL administration, plausibly due to the small sample size associated to wide variability in biomarkers plasma concentrations. Data could be also probably related to the multifactorial nature of the oxidative stress processes and to the need of higher doses of melatonin than those used. Furthermore, no significant effects were found on the prevalence of NEC, BPD, IVH, and ROP in the MEL group than the placebo group. It is noteworthy that the population study represented preterm newborns at medium-low risk to develop these diseases (mean gestational age > than 28 weeks in both groups). Melatonin supplementation in extremely low birth weight or gestational age infants might have a major potentiality to reduce the increase of lipoprotein oxidation products. To our knowledge lack of data exist regarding the valuation of melatonin efficacy in reducing term and preterm

infant morbidity. This study has the limitation of few patients enrolled, and the results need to be confirmed in larger trials. However, the results reported support for the first time the role of melatonin intake to protect preterm newborns against lipid peroxidation. The potential protective role of MEL is mainly due to its beneficial effect on plasma antioxidant status. Moreover, the safety profile of melatonin in clinical study is an encouraging start point for further investigate the protective effects of melatonin on organs and tissues. Our results pave the way for more medical research in this field before melatonin enters in clinical practice. Further research is needed as the schedule that might be effective and the subjects that might receive melatonin to obtain the greatest effect have not been precisely defined.

CHAPTER 5

APPLICATION OF THE OMICS SCIENCES: METABOLOMICS IN PERI-NEONATOLOGY

5.1 Metabolomics: “the new clinical biochemistry” and a tool for future biomarkers

Metabolomics, also identified as "the new clinical chemistry" (Antonucci R, et al. 2010), is a holistic approach based on the systematic study of the complete set of metabolites (metabolome) contained in a biological sample (Mussap M, et al. 2013). It is the most recent of the omics sciences, that focuses on the quantification of the dynamic responses of the metabolome using multivariate analytical approaches derived from genomics methods, a discipline that has now consolidated innovative analysis techniques for situations where the number of biomarkers (genes or metabolites, in the case of metabolomics) far exceeds the number of subjects. The omics sciences are potential key tools for elucidating physiopathological biological mechanisms, for disease screening, for patient risk stratification and, ultimately, for the individualization of medical therapies; compared to the quantifications of transcription factors (transcriptomics) and proteins (proteomics), metabolic quantifications more accurately reflect biological endpoints (Liu J, et al. 2011). In fact, metabolomics deals with simultaneously identifying, quantifying and characterizing thousands of low molecular weight metabolites (<1 kDa), providing a snapshot of the metabolic structure of an individual, that at any moment is a mirror of the physiological or pathological or evolutionary state of the same.

The key point of metabolomics is therefore represented precisely by the concept that the metabolic state of an individual is an accurate representation of the individual's state of health or disease (Mussap M, et al. 2013), but also a dynamic representation, for which the metabolome is considered to be the phenotype that also reflects epigenetic modifications (Fanos V, et al. Adv Clin Chem 2012). With regard to definitions, there are actually two distinct terms, however representing complementary aspects: *metabonomics* is the "measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modifications", while *metabolomics* is "the complete set of metabolites or low molecular weight intermediates, dependent on the context, which vary according to the physiology, state of development or pathology of the cell, tissue, organ or organism ". These two terms are often used interchangeably, although some authors distinguish metabonomics as naturally associated with the whole organism but also intrinsically associated with temporal responses; by analogy, the metabolome is the total repertoire of small molecules present in the cells, tissues, organs and biological fluids under study. Thus, the objectives of a metabolomics study include molecules such as sugars, lipids, small peptides, vitamins, amino acids and organic acids: allowing the parallel evaluation of a wide range of metabolites, metabolomics appears particularly useful for the classification and prediction of physiopathological states through the analysis of the metabolic profiles of biofluids and for the identification of endogenous biomarkers of toxicity (Atzori L, et al. 2009; Antonucci R, et al. 2012). The complexity and size of the metabolome depend on the biological system studied, and we must consider that a metabolome can also be interconnected with another metabolome: for example,

now we know that the interaction of metabolomes of human and intestinal bacterial microflora plays an important role in the state of health and disease of the organism. Until now, no single analytical method has been able to capture the entire metabolome of an organism, however the analytical methods in use (mass spectrometry-MS and nuclear magnetic resonance-NMR spectroscopy) provide the information necessary for the description of the metabolome. (Fanos V, et al. Adv Clin Chem. 2012). The reference for metabolomics studies is represented by the Human Metabolome Database or HMDB (www.hmdb.ca), a metabolomic database available on the web and containing complete information on human metabolites and their biological roles, physiological concentrations, associations with diseases, chemical reactions, metabolic pathways and reference spectra; first described in 2007, HMDB is now the gold standard for human metabolism studies. In just over ten years, the HMDB has presented a continuous evolution in response to the emerging needs of metabolomics researchers and the constant changes in web standards; the latest database update of 2018, HMDB 4.0, is the most significant in its history because it showed an exponential expansion of metabolites and metabolic pathways, as well as research tools and reference spectral data: the most evident change is was the total number of metabolites that, compared to the previous version of 2013 (HMDB 3.0), increased by 5 times from 40153 in HMDB 3.0 to 114100 for HMDB 4.0, corresponding to a 50-fold increase since the first publication in 2007 (HMDB 1.0 , where 2180 metabolites were described). Compounds classified as "detected" metabolites, that is, those with measured concentrations ("detected and quantified") or with experimental confirmation of their existence in biofluids, cells or human tissues even if not quantified ("detected, not quantified") are up to now respectively 18557 and 3271; the "expected" metabolites, i.e. those compounds of known structure for which biochemical pathways are known and in which human uptake or exposure is frequent, but the compound has yet to be detected in the body or the isomer has yet to be formally identified, have switched from 20 641 compounds in HMDB 3.0 to 82 274 in HMDB 4.0 (most of which are lipids) (Wishart DS, et al. 2018).

The magnitude of these numbers gives an idea of the complexity of metabolomics, nowaday facilitated by the available technologies in turn constantly evolving, but that is increased by the fact that living systems, especially in the early stages of life, undergo fast dynamic changes. The basic technology of metabolomics consists of two sequential steps: (1) an experimental technique, based on nuclear magnetic resonance (NMR) analysis and/or mass spectrometry (MS) analysis aimed at creating a compound profile contained in the samples, and (2) a multivariate analysis of the obtained data; results are usually shown graphically in order to highlight the components that contribute to the greatest intrinsic variations of the study groups considered, and the metabolites corresponding to the discriminant variations are then identified (Mussap M, et al. 2013).

Metabolomic analysis of biofluids or tissues has been used successfully in the fields of physiology, diagnostics, functional genomics, pharmacology, toxicology and nutrition, and recent studies have evaluated how physiological variables or pathological conditions can influence the metabolomic profiles of different biofluids in pediatric populations; however, little is known about the general metabolic status

of the full-term and preterm newborn, lacking complete information on the processes of perinatal and neonatal maturation and their metabolic background (Antonucci R, et al. 2010). Introducing new biomarkers for neonatal diseases into clinical practice remains a challenging task, due to the continuous rapid and sudden changes in neonatal physiology during the first month of life (such as changes in body water composition) (Mussap M, et al. 2013), and this fact also applies precisely to the search for biomarkers through metabolomics. However, metabolomics studies relating to neonatal populations are constantly evolving and multiplying, also thanks to a feature of fundamental importance in the neonatal field, that is the respect for non-invasiveness. Indeed, there are several biological fluids that can be used to study the metabolome (urine, blood, saliva, cerebrospinal fluid), even if urine can be considered the best sample - especially for newborns - because it is easy to collect and store and allows precisely a non-invasive approach for the measurement of biological substances; moreover, it can also be considered a complex biological fluid, containing a multitude of biological metabolites and, more precisely, intermediate metabolites, which reflect specific metabolic processes related to health or disease in real time current. Urine can be considered an open window on what happens in the body, representing an ideal "open system" through which various physiological or pathological processes can be observed, such as the balance of water and salt composition, metabolic degradation, the elimination of harmful or toxic substances and the maintenance of homeostasis (Mussap M, et al. 2013). Therefore metabolomics, by tracing the individual metabolic profile intended as the "fingerprint" of each individual at a given time, seems to be a promising tool in Neonatology for monitoring postnatal metabolic maturation, the identification of biomarkers as early predictors of outcome, the diagnosis and monitoring of various diseases and the "tailored" management of neonatal disorders (Antonucci R, et al. 2010; Fanos V, et al. Adv Clin Chem. 2012).

5.1.1 Methodologies and analytical methods

The holistic investigation of the metabolome allows high performing studies at a relatively low cost per sample when compared to other omics sciences such as transcriptomics and proteomics, combining high analytical performance with theory, bioinformatics and computational statistics. Metabolomic studies can be distinguished, based on methodology, in targeted, semi-targeted or non-targeted studies: *targeted* ones study a limited number of predefined metabolites (typically <20) with high levels of specificity, precision and accuracy to define the absolute quantities of each metabolite; the *semi-targeted* methods apply analytical platforms to quantify predefined metabolites, in number equal to a few hundred; *untargeted* methods define the relative concentrations of hundreds-thousands of metabolites precisely but with lower analytical specificity. In the latter case, the metabolites are not predefined and the identification of the biologically relevant ones defining the phenotype (not known a priori) is performed after data acquisition; non-targeted studies offer the greatest opportunity to identify unexpected changes through the application of methods that detect the largest number of metabolites, allow changes in unknown or uncommonly detected metabolites to be observed, and the data obtained provide relative

comparisons between samples (the metabolite concentrations are not reported), unlike targeted studies which provide quantitative data on metabolite concentrations (Dunn WB, et al. 2012).
In other words, the "targeted" approach allows the quantitative analysis of classes of metabolites with a known structure; the "untargeted" approach allows the global analysis of all metabolites present in a biological sample (indeed, it is an investigation capable of revealing any metabolite present in measurable quantities), allowing not only an overview of all known and unknown metabolites, but also the study of the alterations in metabolic pathways that correlate with the different metabolites.
As mentioned above, the metabolomic approach consists of two sequential phases: a first experimental detection technique that allows to measure a large number of metabolites, aimed at creating the profile of the compounds contained in the samples, and subsequently a multivariate analysis of the data obtained (Mussap M, et al. 2013).
Regarding the first phase of the approach, the main analytical techniques used are based on nuclear magnetic resonance (NMR) and mass spectrometry (MS), usually coupled with gas chromatography (GC/MS) or liquid chromatography (LC/MS). NMR spectroscopy can be applied to biological samples, such as urine and blood, with minimal preparation or purification of metabolites and is useful for measuring concentrations with good reproducibility and for non-discriminatory identification, generally used in "open" or " not targeted" approaches; it allows to carry out qualitative and quantitative studies in the context of a complex mixture of metabolites without having to resort to the chemical manipulation of the biological sample under examination. On the contrary, MS can profile xenobiotics and low concentration metabolites in tissues and biofluids (GC-MS allows to distinguish molecules with a very similar structure, LC-MS allows to measure also macromolecules such as lipids or di-tripeptides), but it requires a longer sample preparation, longer analysis times and is less reproducible, more used in "closed" or "targeted" approaches that are limited to a few metabolites (Antonucci R, et al. 2012; Fanos V, et al. Curr Pharm Des. 2012; Pan Z, Raftery D.2007). Going into more detail, proton nuclear magnetic resonance spectroscopy (1H-NMR) is the most widely used technique, allowing the caracterization of low molecular mass compounds containing protons most represented in a biological sample and their representation in a spectrum. 1H-NMR has several advantages, including: (i) high reproducibility, (ii) non-selectivity as regards the metabolites being detected, therefore it allows to simultaneously measure different types of small molecules, (iii) short measurement time, (iv) it allows to analyze small sample volumes from different biofluids, (v) possibility to further investigate unaltered and preserved samples and (vi) detailed information on the molecular structure of metabolites; on the other hand, NMR technique has relatively low sensitivity and NMR spectra can produce overlapping signals between metabolites making absolute quantification difficult. The MS, on the other hand, allows to separate the molecules in a biological sample on the basis of their mass-to-charge ratio and to represent them in a spectrum; this technique (i) is much more sensitive and selective than NMR, allowing for the study of multiple metabolites, (ii) provides reproducible data, and (iii) is applicable to a wide range of analytes, but depending on the paired chromatographic technique, it may have various disadvantages such as (i)

lsample preparation time (e.g. derivatization), (ii) molecule identification and (iii) longer analysis times (Atzori L, et al. 2012).

Despite the differences, both techniques produce a large amount of extremely complex data. The metabolomic analysis of biological samples therefore corresponds to a large dataset, consisting of the measurements of a wide range of metabolites (variables) performed on a number of individuals (observations); multivariate spectroscopic data are typically analyzed using chemometric and pattern recognition techniques, in order to allow sample classification and biomarker identification (Fanos V, et al. Adv Clin Chem. 2012; Antonucci R, et al. 2012 ; Atzori L, et al. 2009). With changes occurring over time or depending on circumstances, metabolites in a dataset frequently show large variations in their absolute concentrations, with a biological significance sometimes being more relevant in percent change rather than in the absolute values; for this reason a pre-processing is commonly performed, in which the data variables are centered and resized with methods deemed most appropriate in each situation. One possible approach (Pareto scaling) sets the mean value of each variable as a zero reference, then scales the quantities in standard deviation units (Liu J, et al. 2011; Yang J, et al. 2015).

The next step is the application of pattern recognition algorithms, that find statistical differences and link them to distinct biological phenomena. There are two distinct approaches to the processing of NMR spectra. In the first approach, known as *chemometry*, no specific metabolites are identified in the spectra, but the spectral patterns and intensities are statistically compared (as in fingerprint analysis); the axes for the variables are divided into "bins" or short intervals, and the intensities for each bin are the statistical variables that are analyzed in order to identify the relevant spectral characteristics that will distinguish different data sets. After grouping the data sets according to their differences, the result is assumed that different classes are distinct and one or more approaches are used to identify and quantify the concentrations of metabolites relevant for each group. With chemometry, therefore, no assumptions are made about the identity and quantity of metabolites in the spectra but, with statistical significance, the spectra are separated into different groups; this is a very efficient approach for large data samples where the compositions and amounts of the metabolites could be very different: chemometry is easily performed, but its results are less easily translated into clinical significance. In the second approach to NMR spectral analysis, known as *targeted profiling*, the first step is to identify and quantify each metabolite in an NMR spectrum, so that the concentrations of the metabolites are the variables, and the next step is the use of statistical methods in order to look for significant differences between them. In targeted profiling it is necessary to have a priori knowledge of the complete NMR spectrum of each metabolite, i.e. it means knowing the spectral position and relative signal intensity for each 1H in each metabolite. In this approach, specific metabolites are therefore quantified and the spectra are distinguished on the basis of them, with necessarily longer times. In situations where the chemical composition of the sample is known, the quantifications use information about the entire peak shape of the metabolite and, depending on the spectral circumstances, will provide more or less accurate quantifications of the metabolite itself (Liu J, et al. 2011). The essential aspect of these techniques is to

calculate, for the purposes of the analysis, a number of factors lower than the data spectrum, which however represent and therefore reflect the same amount of variation present in the larger dataset.
The further next step is represented by multivariate statistical analyzes, that simultaneously consider all the variables in a dataset, describe the correlations between the variables, and generate an image of the overall changes in the metabolic network using two main approaches (Atzori L, et al. 2012): "unsupervised" approaches such as Principal Component Analysis (PCA), and "supervised" approaches such as Discriminant Function Analysis (DFA) or Partial-Least Squares-Discriminate Analysis (PLS-DA). PCA transforms an authentic set of associated variables variables into a new set of unrelated latent variables, called "principal components"; this method allows to identify any intrinsic clusters of the sample that may be discriminated through their spectral characteristics. The supervised PLS-DA approach, on the other hand, is a regression technique to model the relationship between projections of dependent factors and independent responses; the supervised mode of pattern recognition techniques creates mathematical models that are then used to test an independent data set (Atzori L, et al. 2009). Practically, these mathematical procedures are "calibrated" to to spotlight inner similarities among samples, create groups and highlight differences between detected groups. The models obtained with these procedures are then tested to be validated and optimized (search for outliers) to evaluate their robustness and predictability performance on unknown data sets; the aim is to identify, among all the variables produced by the metabolomic analysis, the significant ones (covariates) for the best separation of the groups of interest. Indeed, given the multivariate nature of biological data, the main information resides in the common alterations of the variables, defined as pattern correlation between the observations, and it is possible to perform mathematical transformations on the data themselves, from the original space of the variables to the metabolomic one, selecting the highly-correlated and significant alterations. In this way it is possible to define new sets of variables called "scores", expressed as a combination of the original ones with the important property of not being correlated: these new sets of variables, defined as "principal components" or "latent components", have the relevant property of being able to describe the underlying trends in the data; the correlations that the principal or latent components have with the original variable are called "loadings". The mathematical transformation of the data produces the same number of latent variables as the original, but the underlying trends are usually described by a smaller number of variables; therefore, in multivariate analysis, only a subset of variables contains useful information, and we can identify these variables by finding correlation patterns among the data. Furthermore, multivariate spectra analysis can be used to reduce the dimensionality of the data without loss of information by simply removing the variables that can be considered "noise" for the problem of interest (Fanos V, et al. Adv Clin Chem. 2012) . The graphical representation of the analyzed variables is represented by corresponding "scores plots" (clusters of variables) and "loadings plots" (identifying the metabolites that contributed most to the separation of the clusters in the scores plots) (Liu J, et al. 2011); the comparison between loadings plots and scores plots allows to establish which variables are relevant for the analyzed data. Today there are computer platforms available that facilitate

the integrated visualization of many different types of omics data, such as PaintOmics 3, a web tool that allows the complete exploration of multi-omics data via path models: using this tool, researchers can easily move through different levels of regulation within biological systems by exploiting the combined advantages of network graphs to study global path relationships and multi-omic graphing maps to understand molecular interactions (Hernández-de-Diego R, et al. 2018).

Figure 7 below illustrates the workflow of metabolomic processes.

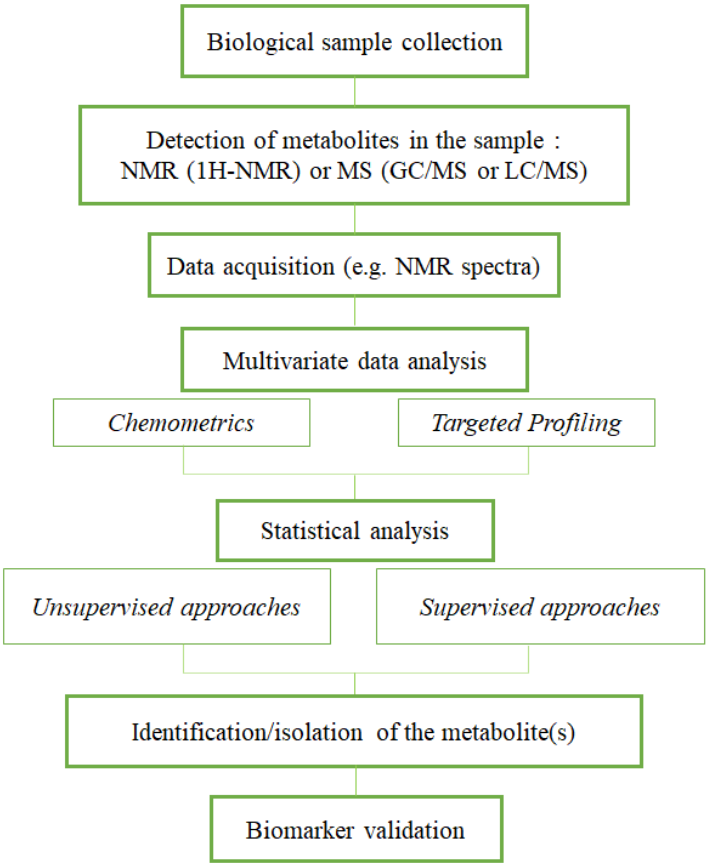

Figure 7. Workflow in metabolomics studies

5.1.2 Clinical applications

At present, improve knowledge of perinatal "programming", identify short- and long-term effects of prematurity or delivery mode, shed light on early brain damage, discover early non-invasive diagnostic biomarkers and predict the type of response to pharmacological or non-pharmacological treatments represent some of the most difficult challenges in neonatology (Fanos V, et al. J Matern Fetal Neonatal Med. 2012). We have already seen above that the maternal-fetal-environmental interaction is a much debated issue in perinatology and neonatology, as well as the first moments of post-natal life that represent the transition and adaptation phase to extrauterine life; moreover, many peri-neonatal conditions (for example prematurity-related diseases) and early interventions (for example nutritional, pharmacological, and neurobehavioural ones) impact on the long-term health of each individual: metabolomics, providing an instantaneous of the state metabolic and therefore a sort of fingerprint of

the individual, represents a potentially useful tool to describe the physiological or physiopathological variations that contribute to the establishment of certain conditions under study. This is the reason why metabolomics research in the prenatal and peri-neonatology field is in massive expansion: using exclusively search terms "metabolomics" and "newborn", about 1000 results can be found in Pubmed, but with an exponential increase in the last decade. The fields of application of metabolomics are multiples; in particular in the obstetric and neonatological-pediatric context, investigations are increasing relative to pregnancy and fetal diseases such as GMD, IUGR, pre-eclampsia, but also to preterm birth, perinatal transition, peri-neonatal asphyxia and neonatal brain damage, postnatal maturation and correlation with biological and chronological age, differences related to delivery methods and gestational age, prematurity.related diseases such as PDA, RDS, BPD, NEC, kidney pathology/nephrouropathies, inborn errors of metabolism or metabolic diseases, sepsis, long-term outcome of VPTs and EPTs. Metabolomics is also being studied with regards to clinical responses to drugs (pharmacometabolomics) and nutrition (nutrimetabolomics, including breast milk assessment) (Fanos V, et al. J Matern Fetal Neonatal Med. 2012; Adv Clin Chem. 2012; Curr Opin Pediatr. 2013; Neonatology. 2018).

Regarding *gestational diabetes mellitus*, a recent review has shown that many of the studies reviewed found significant differences in amino acids and related metabolites, fatty acids and related metabolites, phospholipids and bile acids among women with GDM and without GDM, however the results are strongly contradicting between the studies about the direction and the clinical significance of specific metabolites (Chen Q, et al. 2018). *Intrauterine growth retardation*, IUGR, is a complex disease and information regarding the metabolic profile of IUGR is fragmented despite being one of the most common problems in the care of preterm infants. Metabolomics was applied in a study involving two groups of newborns: the first group consisting of preterms with IUGR diagnosed by fetal ultrasound; the second group consisting of adequate-for-gestational age preterms as controls. Urinary samples were collected at birth and analyzed by 1H-NMR: the analysis of the two populations was significantly different as regards the urea cycle, and the metabolism of amino acids glycine, serine and threonine, resulted increased in the IUGR group; the discriminants in urine metabolic profiles are essentially derived from significant differences in some metabolites such as myoinositol, sarcosine, creatine and creatinine. The metabolomic analysis therefore showed different metabolic profiles of the urine between preterm with IUGR and controls and allowed to identify the molecules responsible for these differences (Dessì A, et al. 2011); in accordance with this result, several published studies on animal models of IUGR, neonatal population or cell cultures have shown an increase in the level of myoinositol in placenta, fetal arterial plasma, and fibroblasts: although the role of myoinositol is not yet clear, it appears to be associated with the later development of the metabolic syndrome (Mussap M, et al. 2013; Fanos V, et al. J Matern Fetal Neonatal Med. 2012; Adv Clin Chem. 2012).

Preterm birth (PTB) is also an interesting field of research, because the metabolomic characterization of biofluids collected during prenatal period offers the possibility of detecting peculiar metabolic

patterns prior to the PTB itself, constituting early predictive biomarkers able to guide therapeutic decisions. The metabolomic studies analyzed, on the one hand, prenatal biofluids (such as amniotic fluid, urine or maternal blood, cervicovaginal fluid) to identify predictive biomarkers of PTB and, on the other hand, biofluids collected during or after birth (amniotic fluid, cord blood, neonatal urine and blood, maternal blood or breast milk) to assess and monitor the health status of infants with PTB. With regard to the predictivity of the event, changes in metabolic profiles were observed depending on the studies and the trimester of pregnancy investigated (Gil AM, Duarte D.2018); further systematic reviews and trials are underway regarding the application of metabolomics in the prediction of PTB (Cecatti JG, et al. 2016; Souza RT, et al. 2019).

Regarding the differences between *full-term and preterm infants*, it was possible to reveal distinct metabolic patterns associated with different gestational age groups, suggesting that the metabolic status of the newborn at birth is strictly dependent on its GA. The most important discriminating compounds between different classes of newborns were also identified: the metabolic pathways involved were found to be the metabolism of tyrosine; the biosynthesis of tyrosine, tryptophan, phenylalanine; the urea cycle; arginine and proline metabolism. In particular, Atzori et al. identified hippurate, tryptophan, phenylalanine, malate, tyrosine, hydroxybutyrate, N-acetyl-glutamate and proline as discriminating metabolites (Atzori L, et al. 2011). In a preliminary cross-sectional study, some Authors explored differences in urinary metabolome between preterm <32 weeks GA and full-term infants versus adult subjects: significant differences in urinary metabolome were evident in urine samples collected early after preterm and term birth, with significant differences in the urinary metabolites alanine, formate and citrate (higher in preterm infants) and creatinine, creatine and dimethylglycine (lower in preterm infants). Metabolic differences between term and preterm infants persisted until term equivalent age (MJ Hyde, et al. 2010) and these data are suggestive for persistent alterations in metabolic pathways (Fanos V, et al. Adv Clin Chem. 2012). A recent work reported an NMR-based metabolomic study of neonatal urine samples to investigate the metabolic impact of prematurity, compared to other neonatal disorders such as RDS, being large for gestational age (LGA) and/or suffering from malformations: results showed that gender and delivery mode have a significant impact on urine composition and, therefore, must be taken into account in metabolomic approaches; furthermore, preterm births were characterized by a wide variability of metabolites, indicative of disturbances in nucleotide metabolism, in the biosynthesis of pulmonary surfactant and in renal function, and by an increase in the activity of the tricarboxylic acid (TCA) cycle, oxidation of fatty acids and oxidative stress. Significant metabolites specificately related to prematurity include indoxyl sulfate (IS), 3-hydroxyisovalerate (3-HIVA) (↑), dimethylamine (DMA) (↓) and 1-methylhistidine (↓) (Diaz SO, et al. 2016). Alexandre-Gouabau et al. used a MS-based chemical phenotyping approach to study the overall effect of preterm birth on fetal metabolism and maternal-fetal nutrient transfer. Venous and arterial umbilical blood and blood from mothers who have undergone preterm labor or delivered full-term neonates with very low birth weight were sampled: a significant increase in maternal-fetal levels and gradients of butyryl-, isovaleryl-, hexanoyl- and

octanoyl-carnitines was found in the VLBW group, suggesting an increase in beta-oxidation of short- and medium-chain fatty acids in the preterm fetal-placental unit. Furthermore, significantly reduced levels of glutamine-glutamate in preterm arterial cord blood, as well as lower concentrations of Krebs cycle amino acid precursors, appear to indicate increased glutamine utilization in the preterm fetus. Finally, an increase in both circulating levels and maternal-fetal gradients of various acetylated polyamines suggests an increase in the metabolic cycle of polyamines in conditions of extreme prematurity. This confirms that alterations in fetal energy, antioxidant defense, and the flow of polyamines and purines are a metabolic signature of prematurity (Alexandre-Gouabau MC, et al. 2013; Fanos V, et al. 2013). However, the studies performed to date on metabolomic predictors of preterm birth are highly heterogeneous both in methodology and in the resulting identification of metabolites, so there is an urgent need for larger studies in well-defined populations to determine predictive biomarkers of preterm birth and to reveal mechanisms and objectives for the development of intervention strategies (Carter RA, et al. 2019).

Through the metabolomic approach, *age-related metabolic changes* were also investigated. Pathways related to amino acid metabolism appear significantly different in children aged 6 months to 1 year, while those associated with carbohydrate metabolism were significantly different among children aged 2 to 3 years (Chiu CY, et al. 2016). The analysis of urine samples using 1H-NMR spectroscopy, in children 12 years of age and younger, have shown that apparently age-related metabolites include creatinine (increased), creatine, glycine, betaine/TMAO, citrate, succinate and acetone (all reduced); these results can potentially be useful for assessing the biological age (as a parameter distinct from chronological age) of children (Gu H,, et al. 2009; Fanos V, et al. Adv Clin Chem. 2012).

Regarding the influence of *delivery mode*, elective cesarean section (CS) without labor was shown in one study to correlate with lower levels of isoleucine, fructose, mannose, glucose, allose, glucuronic acid, inositol and cysteine compared to spontaneous vaginal delivery (SVD) after spontaneous labor without use of drugs: it has been hypothesized that the stress associated with labor is involved in alterations of the levels of metabolites, in particular of saccharides such as glucose, in the umbilical cord blood (Hashimoto F, et al. 2013). It has recently been reported that the urinary metabolic footprints of infants born by spontaneous delivery or CS reflect important short-term adaptations of metabolism: differences in thermogenesis and perturbations in energy metabolism at birth in those born from CS have a profound influence on biochemical pathways involved in the oxidation of fatty acids, in gluconeogenesis, in the biosynthesis of surfactants and in renal functions. Indeed, those born by SVD showed significantly higher urinary levels of two dicarboxylic acids (suberate and sebacate), of an intermediate of the Krebs cycle (oxaloacetate) and of lactate, and this suggests a different metabolism of oxidation of fatty acids; they also showed lower urinary levels of hippurate, histidine and lysine, but higher levels of alpha-aminoadipate (a precursor of lysine), lower urinary content in the osmolyte choline, but greater urinary excretion of inositol, and greater urinary excretion of N-acetyl-glycoproteins compared to children born by CS (Martin FP, et al. 2018). It has also been shown that gut microbiota

and faecal metabolite composition are significantly different between SVD and CS infants (Li N, et al. 2021).

One of the main fields of application of metabolomics is represented by *perinatal asphyxia* and *hypoxic-ischemic encephalopathy* (HIE), in relation to which just under 30 works have appeared in literature in the last two decades, most of them conducted on animal models but also recently on human models: urine and/or cord blood were studied, evaluating the differences between asphyxiated infants and healthy controls or, in the asphyxiated population, between favorable or unfavorable neurological outcome. Overall, a different metabolic fingerprint was observed in infants with negative outcome compared to those with better prognosis and healthy controls; moreover, it has been reported that asphyxiated infants with a favorable outcome show a metabolic profile that over time approaches that of healthy controls, while asphyxiated infants with a poor prognosis maintain a peculiar metabolic profile (Marassi MLG, et al. 2021). Chu et al. showed that high concentrations of 8 urinary organic acids in distinct biochemical pathways were significantly associated with the prognosis with a high sensitivity and specificity: in particular, ethylmalonate, 3-hydroxy-3-methylglutarate, 2-hydroxy-glutarate and 2-oxo-glutarate were found to be associated with a good outcome, while glutarate, methylmalonate, 3-hydroxy-butyrate and orotate correlated with an unfavorable outcome (Chu CY, et al. 2006). More recently, it has been shown that metabolites related to better prognosis are linked to the Krebs cycle (acetate, citrate, α-ketoglutarate) (Noto A, et al. 2016; Locci E, et al. 2018) or act as osmolytes (myoinositol, choline, betaine, taurine) or in the formation of cell membranes; ketone bodies (acetoacetate and β-hydroxybutyrate) seem to have an important role in the prevention of neurological damage, as their levels are reduced in infants with HIE compared to healthy controls (Ahearne CE, et al. 2016).

As regards prematurity-related diseases, in the case of *RDS* some overexpressed metabolites during invasive ventilatory support and after treatment with surfactant have been highlighted in the bronchoalveolar lavage fluid (Fabiano A, et al. 2011), but to date the metabolomics research of predictive biomarkers of *BPD* is particularly active. From the comparison between VLBW preterms that subsequently developed BPD and preterms that did not develop BPD, five important discriminating urinary metabolites emerged: indeed, in the former there were increased lactate, taurine, TMAO, and myoinositol levels and reduced gluconate levels (Fanos V, et al. 2014). The group of Atzori and Fanos observed that the discriminating metabolites between the two groups were alanine, betaine, trimethylamine-N-oxide, lactate and glycine; using metabolomics, it was possible to fingerprint the urinary metabolic pattern in the first week of life of infants who subsequently developed BPD (Pintus MC, et al. 2018). Another recent study evaluated 160 tracheal aspirate samples collected in the first week of life from 68 preterm infants, including 44 with subsequent BPD and 24 without BPD: a cluster of 53 metabolites was identified as characteristic of BPD, with 18 selected metabolites highly significant in the differentiation between BPD and no-BPD. Levels of amino acids histidine, glutamic acid, citrulline, glycine and isoleucine were higher in infants with BPD, as were acylcarnitines C16-OH and

C18:1-OH (especially elevated in infants with GA <27 weeks). Metabolomics therefore appears to be a promising tool for the early identification of predictive-prognostic biomarkers that, at least as regards BPD, can help guide the subsequent therapeutic process (Piersigilli F, et al. 2019).

The metabolomic pathways that characterize urine samples collected in the first 12 hours of life of preterm infants also seem to correlate with the probability of developing a *hemodynamically significant PDA* (hsPDA), confirmed at 48-72 hours of life. In fact, a strong difference has been reported between the metabolomic profile of those infants who subsequently show hsPDA and no-hsPDA infants: the hsPDA group was characterized by lower levels of 3-methylxanthine, betaine, glycylproline, TMAO, tryptophan, myoinositol and 4- hydroxyproline and higher glucose and lactate levels (Bardanzellu F, et al. 2020).

Another application of metabolomics regards *nephro-uropathies*. Some Authors have manegd to discriminate infants with nephrouropathies (renal dysplasia, vesicoureteral reflux, urinary tract infections and acute kidney injury) from healthy infants by means of the urinary metabolic profile, with differences linked to alterations in purines and pyridines and in the urea cycle (Atzori L, et al. 2010). Metabolomics also appears to be a promising tool for the diagnosis of acute kidney injury (AKI) in neonatal and pediatric age (Mussap M, et al. 2014; Mercier K, et al. 2017; Muhle-Goll C, et al. .2020); indeed, specific metabolites have been identified characterizing the metabolic profile of newborns with AKI (1-methylnicotinamide, 2-hydroxybutyrate, 4-hydroxyphenyl-lactate, acetic acid, ethanolamine, ethylene glycol, N-acetyltyrosine, acetylphosphate, propylene glycol, dimethylamine, glucuronate) and that of infants without AKI (uridopropionic acid, creatine, dimethylamine, glutamine, glycine, leucine, methionine, myo-inositol, succinylacetone, trans-4-hydroxy-L-proline, 4-hydroxyphenylactate, creatinine, hippurate, glycolate, glutathione, isoleucine, glutamate) (Mercier K, et al. 2017).

Metabolomics can aid in the clinical management of *sepsis*: interactions between metabolism and the immune response are increasingly recognized, as changes in metabolic pathways drive the function and activation of innate immune cells and consequently the host response to pathogens. Septic infants demonstrated metabolic profiles distinct from age-matched non-septic controls, such as higher amounts of glucose, pyruvate and lactate and lower levels of glutamine and B-complex vitamins, such as riboflavin (vitamin B2) and nicotinamide (vitamin B3) (Conti MG, et al. 2020).

An expanding sector also in neonatology is represented by *pharmacometabolomics*, aimed at predicting the metabolism and toxicity of a drug on the basis of the analysis of a pre-dose metabolic profile, at identifying drug-related alterations in the metabolic pathways and at discovering the mechanisms underlying sporadic idiosyncratic toxicity: drug-induced responses in individuals are in fact potentially predictable from their pre-dose metabolic profiles, which act as biochemical signatures that reflect relevant factors to drug metabolism and drug effects. In practice, the drug-metabonomic concept is based on the ability to predict a post-intervention metabolic profile starting from a pre-intervention metabotype (Fanos V, et al. Curr Opin Pediatr. 2013; Curr Pharm Des. 2012).

Finally, metabolomics is the logical approach to evaluate the complex relationship between nutrition and metabolism, the roles that dietary components play in many aspects of health and disease and how the human metabolic balance can be disturbed by nutritional deficiencies or excesses: therefore, *nutrimetabolomics* can become one of the main applications of metabolomics, particularly from the early stages of development and in the first years of life; from a theoretical point of view, the newborn is a perfect candidate for nutrimetabolomic studies, since his diet is very strict. However, differences were observed in the metabolic patterns of breast-fed or formula-fed infants (with differences also between different types of formula) (Cesare Marincola F, et al. 2016). The various types of milk were also analyzed using metabolomic approaches: breast milk showed higher concentrations of lactose than formula milk, that on the contrary had higher concentrations of galactose 1-phosphate and maltose. Breast milk of mother of very premature infants (23-25 weeks) exhibited a different metabolic profile compared to that of mother of preterm infants ≥29 weeks, with a subsequent tendency to similarity only around the 30 weeks postnatal age; breast milk of preterm infants at 29-34 weeks, collected up to 40 weeks of postnatal age, showed a temporal variation in the first three weeks of breastfeeding (Longini M, et al. 2014). Subsequently it was observed that the metabolomic pattern of preterm milk does undergo maturation during the first 3 weeks after birth, but at the end of the third week it still does not resemble the metabolic pattern of term milk: the specific changes in the metabolomic profiles of breast milk based on the characteristics of their offspring could reflect the different nutritional needs of each preterm infant, and this knowledge is essential to move from standardized nutritional protocols to a personalized nutrition in preterm infants (Perrone S, et al. Nutrition. 2019).

Finally, an important application of metabolomics concerns the long-term follow-up of EPTs: some Authors were able to discriminate a population of adults who were born preterm with extremely low birth weight (ELBW) from a control population of born full-term adults. Differences in the two groups were related to alterations in arginine and proline metabolism, purine and pyrimidine metabolism, histidine β-alanine metabolism, and the urea cycle (Atzori L, et al. J Matern Fetal Neonatal Med. 2011). Differences in metabolome between ex-term and ex-preterm were also observed in young adults by other Authors: the most marked differences were found among young adults born preterm regarding increased methylamines and acetyl-glycoproteins and lower hippurate levels compared to young adults born at term (N.Modi, et al. 2010; Fanos V, et al. Adv Clin Chem. 2012).

5.1.3 Metabolomics and oxidative stress

Since metabolites are the downstream end products of gene expression and are affected by epigenetic modifications and therefore by environmental factors, metabolomics provides a “phenotypic signature” of the physiopathology of the diseases using a minimum amount of body fluids. Considering the etiopathogenetic relevance of oxidative stress in prematurity-related diseases, it seems understandable that metabolomics can represent a useful tool aimed at investigating this causal link and providing biomarkers of OS. Since OS determines changes in multiple cellular and tissue structures, the study of a biomarkers' panel rather than a single biomarker is generally more indicative, but as mentioned above,

there are still limits such as the need for specific kits and tools to measure OS biomarkers, and consequently the need for an experienced laboratory team and high costs. Metabolomics could provide OS biormarkers' panels in a relatively short and affordable time and cost: multivariate analysis techniques should allow to discern the characteristics of the sets of metabolites that distinguish different physiological processes and, in so doing, to show key aspects in the physiopathology of diseases and therefore develop useful and targeted interventions. The goal of studying OS metabolomics is to develop methods that serve as a tool for diagnosis, treatment guidance, and personalized drug discovery in clinical conditions in which OS plays a prominent role (Liu J, et al . 2011), as in the case of prematurity. Due to the complexity and unreliability in measuring FRs, downstream products reflecting a failure of cellular antioxidant capacity leading to oxidative damage are ideal metabolomic targets for assessing OS; some Authors are developing metabolomic methods applicable in vivo and in vitro for the study of biomarkers of OS, in particular lipid products (isoprostanes) (Schoeman JC, et al. 2018) or antioxidants and other metabolites (malate, vitamin c, reduced glutathione GSH and tryptophan) (Wang N, et al. 2016). In the review by Liu et al. a wide variety of publications have been found that report clinical and mechanistic associations between OS and specific metabolites, groups of metabolites and metabolomic analyzes, however mostly concerning areas of adult interest (Liu J, et al. 2011). In the peri-neonatal field, the most expanding clinical research area is probably that of perinatal asphyxia and related hypoxic-ischemic encephalopathy (Liu J, et al. 2011; Marassi MLG, et al. 2021; Solevåg AL, et al. 2021).

As regards *FRD*, prematurity-and-oxidative stress related pathologies, metabolomic studies begin to appear in the literature that indirectly document a role of OS in these conditions, identifying as discriminating some metabolites involved in inflammatory processes and oxidative damage. This is particularly relevant for defining the metabolic patterns, or rather their deviations, which over time predispose to future health problems (such as metabolic ones, in accordance with the Barker hypothesis) typical of preterm and low weight infants. Some Authors, who have conducted metabolomic studies based on LC-MS on cord blood and maternal blood of preterm VLBW infants, have observed models concerning acetylcarnitine, glutamine, glutathione and nitric oxide that are consistent with the increased use of fatty acids such as energy source, the increased use of glutamine and an altered oxidative stress, all conditions previously known to be associated with VLBW and that therefore represent a signature of prematurity (Alexandre-Gouabau MC, et al. 2013). In another study, mentioned above, premature infants were characterized by alterations in various metabolites indicative of disturbances in nucleotide metabolism, surfactant biosynthesis and renal function, and by an increase in the activity of the tricarboxylic acid cycle, the oxidation of fatty acids and oxidative stress; comparison with other pathological conditions has shown that this profile appears to be largely specific to premature infants, ie of the same prematurity. Indoxyl sulfate (IS) is known to mediate OS in endothelial cells of the human umbilical vein: higher IS levels found in premature infants may reflect increased OS, although it may also be related to impaired renal function (Diaz SO , et al. 2016). In a recent study, a clear metabolomic

signature of the low birth weight (independent of the GA) was also reported as detectable in adolescence, adulthood and old age in three independent cohorts of subjects. 4 types of compounds have been identified (acylcarnitine, gamma-glutamyllucine, hydroxyphenylactate and alpha-hydroxybutyric acid) for which higher levels in adulthood appear to correlate with low birth weight: acylcarnitines and alpha-hydroxybutyric acid are directly linked to insulin resistance; higher levels of gamma-glutamylleucine and alpha-hydroxybutyric acid are both associated with oxidative stress, and hydroxyphenyl-lactate appears to be a biomarker of mitochondrial dysfunction. This evidence seems compatible with the Developmental Origins of Health and Disease (DOHaD) hypothesis, but it also underscores the critical role of lower insulin sensitivity and impaired energy metabolism in linking the early energy deprivation to a poor health outcome in old age (Metrustry SJ, et al. 2018).

Finally, one of the most relevant metabolites described in neonatology and related to OS is glycine, the smallest amino acid used by the body to synthesize various biologically relevant compounds, such as glucose, and elements that are part of both the defense system against oxidative stress and the detoxification reaction, having a key role in the synthesis of glutathione (GSH). High levels of glycine in the urine collected in the first week of life of SGA and IUGR infants, as well as LGA, have been described in the literature: this seems to reflect their altered metabolic state, mainly catabolic state, with a weakening of the defense systems capable of preserving the organism from the OS and the related pathologies. Furthermore, glycine levels have been found to be increased in the urine of newborns with NEC, and since glycine is stored in intestinal cells it could be a possible biomarker of OS-related intestinal damage (Fanos V, et al. Neonatology. 2018).

5.2 "*Newborn metabolomic profile mirrors that of mother in pregnancy*" (Published on Medical Hypothesis 137 (2020) 109543)

5.2.1 Introduction

Pregnancy is characterized by a complexity of metabolic processes that may impact foetal development and infant health outcomes (Lindsay KL,et al.2015). Understanding the changes in maternal metabolism before, during and after pregnancy is an essential clue regarding future neonatal health. In agreement with the "Barker hypothesis", there are many elements which may affect both the smooth progress of a pregnancy and the foetal and neonatal outcome (Fall CH.2013; Osmond C, et al.1993; Barker DJ,et al.1993). Metabolomic is one of the techniques that best allows investigation of complex biological systems (Noto A,et al.2016). Metabolomic technology, measuring multiple metabolites, directly from biological systems, offers enormous potential to discover changes in maternal metabolism during pregnancy and their relation to the newborn metabolic status (Hellmuth C, et al. 2019). Recently, metabolomics has found a strong field of application in perinatology. Many studies have demonstrated that metabolomics is a powerful method for detecting detailed metabolic signature of healthy pregnancies adding an important step towards the identification of disease-related deviations of the major obstetric pathologies (Horgan RP,et al. 2011; Beecher CW.2011; Orczyk-Pawilowicz M,et

al.2016). Diaz et al. observed a correlation between metabolomic profiles and foetal malformations (Diaz SO,et al.2013). Other authors investigated metabolomics in newborns from mothers with severe preeclampsia (Woodham PC, et al. 2012; Kenny LC,et al.2010; Bahado-Singh RO, et al.2013), in pregnancies with small for gestational age (SGA) foetus (Horgan RP,et al. 2011) and in pregnancies delivering preterm newborns (Beecher CW.2011). Nevertheless, current understanding of the relationship between the metabolomic profile of mother and newborn during normal fullterm pregnancy is still not complete. The present study aims to investigate the correlation between urinary metabolomic profile of healthy mother and their newborns. We tested the hypothesis that the newborn metabolomic status is associated to that one of its mothers, in the last trimester of pregnancy.

5.2.2 Materials and methods

Population

The study was carried out at the Department of Molecular and Developmental Medicine, University of Siena, Italy. Institutional Review Board approved the study. A total of 57 urine samples were collected: 36 from pregnant women, three weeks before delivery, and 21 from healthy newborns, within 48 h of birth. Maternal and newborns'clinical details are reported in Tables 3 and 4 (corresponding to tables 1 and 2 of the published article attached as Annex 11). All of the urine samples were collected after written informed consent obtained from the individual women. The decision to use urine as the target of the investigations has been dictated by the need to make this study non-invasive and to facilitate adherence by patients. Regarding the mothers, the inclusion criteria were: -obtaining a free and informed consent from the pregnant women; -achievement of at least 36 weeks of gestational age (GA) at the time of urine sample collection; -time of delivery between 37 and 42 gestational age. The exclusion criteria were: -pregnancies with a gestational age<37 weeks; -twin pregnancies.

Table 3 Clinical characteristics of pregnant women.

Gestational Age (weeks) at sample collection, mean (SD)		37 (1)
Mother's age (years) at delivery, mean (SD)		33 (4)
Weight gain (Kg) during pregnancy, mean (SD)		15 (5)
Maternal Gestational Diabetes, n (%)		4 (11)
Maternal Hypertension, n (%)		2 (6)
Maternal Hypotiroidism, n (%)		2 (6)
PROM, n (%)		9 (25)
Positive vaginal swab, n (%)		6 (17)
Type of delivery, n (%)	Vaginal	23 (64)
	C-Section*	13 (36)

Table 4 Clinical characteristics of newborns

Gestional Age (weeks), mean (SD)	39 (1)	
Birth Weight (gr), mean (SD)	3370 (601)	
Small for Gestational Age, n (%)	-	
Large for Gestational Age, n (%)	4 (19)	
Gender, n (%)	12 Male (57)	9 Female (43)

Urine samples

Neonatal urines were collected by the insertion of a cotton pad inside the diaper. As regards to the collection of neonatal urine samples, no exclusion criteria were adopted if not the refusal to consent to the collection of the sample. Each patient, pregnant women and newborns, provided a single sample during the study. All urine samples were shipped in dry ice to the Laboratory of the University of Siena. The samples were then analysed using a nuclear magnetic resonance spectroscopic (Nuclear Magnetic Resonance, NMR) analysis technique.

NMR analysis

Urine NMR measurements were performed on a Bruker DRX 600 MHz Avance Spectrometer with a selective inverse probe (SEI) equipped with Z gradient coil. Spectra were acquired at a constant temperature of 298.0 ± 0.1 K by using 90° pulses. Furthermore, 10 s delay was included in the pulse sequence to allow T1 relaxation. In fact,

T1 values (in the range 1.5–2.8 s) of the analysed metabolites are such that a 10 s delay allows full recovery of longitudinal magnetization after a 90° pulse, as verified by constant integral values for D1≥5 s. A 0.3 Hz line broadening function was applied before Fourier transformation. A saturation pulse of 2 s duration was applied at the water resonance to suppress the water signal. 32 K data points per scan were used, and 128 transients were accumulated. Each urine sample was first centrifuged at 2000 rpm for 5 min and analysed afterwards. Sample (550 µl) plus 50 µl of a TSP-d4 20mM solution were measured into a 0.5mm (outer diameter) MR tube. All spectra were first run at their own physiological pH; we use this first spectrum only for an overview of the contained metabolites; then, we adjust the pH at 2.50 ± 0.02 in the same MR tube, with a microelectrode, and we run a second spectrum.

The chemical shift of ionizable fluids is highly dependent on the pH. At a pH of 2.50, all chemical shift values are reproducible within ± 0.01 ppm (Wevers RA, et al.1999). Moreover, under the described conditions, the methyl signals of creatine and creatinine are clearly separated (3.05 ppm for the methyl signal of creatine and 3.13 ppm for creatinine) and the methyl signal of lactic acid (1.41 ppm) is not overlapped by the methyl resonance of threonine (1.33 ppm). The pH was adjusted using a minimal volume of HCl, starting from a 3M and ending with a 0.05 M, and samples were directly frozen at −80 °C (Tataranno ML,et al.2018). All samples were run at the same time. The variables of interest, related to the collected samples, were described in a dataset containing multiple clinical data of patients enrolled in the study.

Statistical analysis

The spectra were examined by analysis of the main components (PCA), through complex computer processing systems, based on anamnestic and clinical data (collected in the dataset) relating to both pregnant and newborn. The PCA is the first statistical approach to a metabolomic analysis and is aimed at finding a trajectory or a possible cluster formation in the study samples on the Cartesian plane, also called score plot. At first, a technique was used for data simplification, as the variables studied were multiple. This technique allows to obtain a linear transformation of the variables that projects the original

ones into a new two-dimensional Cartesian system in which the new variable with the greatest variance in data is projected on the horizontal axis (values of the principal component 1, PC 1), while the second variable for variance size, called principal component 2 (PC 2) on the vertical axis. Then, a multivariate regression system was used, with the aim of making quantitative predictions relative to one or more properties of the spectra in question, using the Partial Least Square (PLS). PLS is a further development of the PCA, as the components used are derived from the set of PCA responses. In this way it is possible to maximize the variance not only of PC 1 (abscissa) but also of PC 2 (ordinate). In doing so, the choice of factors (main components), to be used for analysis, is even more focused and effective. The PLS allows to better balance the information contained in the abscissae and the ordinates on the score plot, reducing the effect of large but irrelevant variations between the data provided.

5.2.3 Results

Figure 8 reports the median spectrum relative to maternal urine (A; corresponding to Fig. 1A of Annex 11) and the median spectrum relative to neonatal urine (B, corresponding to Fig. 1B). The first component of the PCA analysis (PC1) showed two distinct metabolic groups: pregnant women and newborns (figure 9, corresponding to Fig.2 of the article below). Among all enrolled samples (n=57), 14 were pairs: a mother and her own baby. A significant correlation was found between urine metabolic profiles of mothers collected 3 weeks before delivery and those one of their newborns collected after birth, as shown through the scores of the second component (PC2) of the PCA analysis (figure 10, corresponding to Fig. 3 of the article). In figure 10 each square corresponds to a pair (mother-child) which a specific colour has been assigned within the scatter plot.

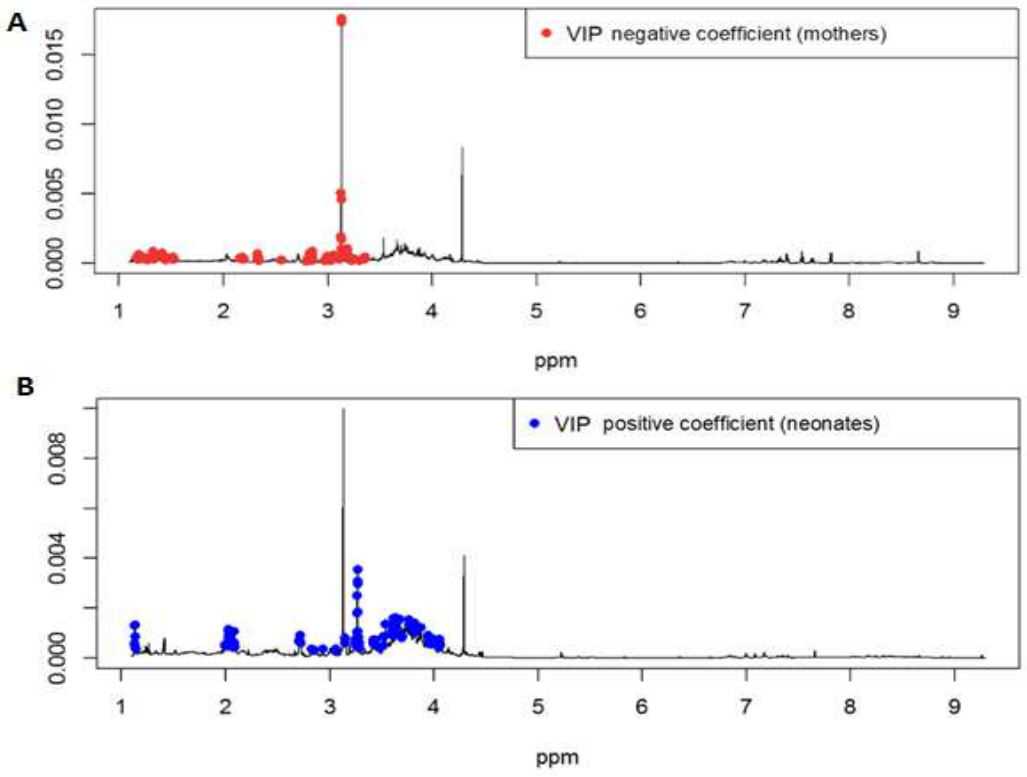

Figure 8. Median spectrum of mothers (A) and newborns (B); VIP (Variable Influence in Projection), ppm (parts for million).

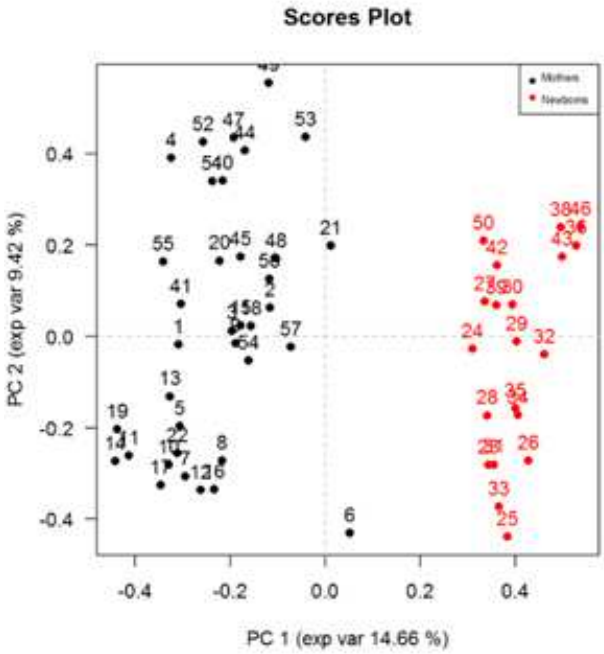

Figure 9. The first component of the PCA analysis (PC1) of urine metabolic profile of pregnant women (black dots) and newborns (red dots). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

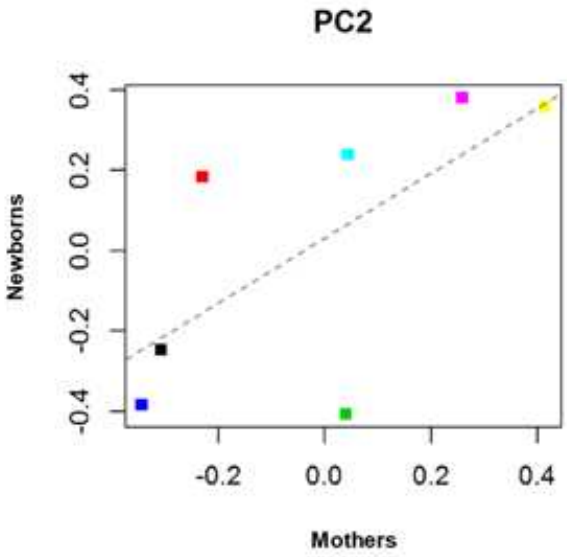

Figure 10. Analysis of the 14 pairs: mothers and their own baby. Correlation between urine metabolic profiles of mothers collected 3 weeks before delivery and those one of their newborns collected after birth.

5.2.4 Discussion

Principal findings of the study

The principal findings of the study are as follows: 1) analysis of the urine spectra showed that the metabolic profile of pregnant womens is different from that of newborns, 2) the PCA analysis demonstrated that urinary metabolic profile of pregnant mother, three weeks before delivery significantly correlated with metabolic profile of their own babies, 3) there are several metabolites that help to highlight the correlation in the profiles of the mother and her child. These metabolites are a powerful link between the two profiles. The study provides, for the first time, a comparison of the metabolomic profile of mother and newborn during normal full-term pregnancy. Already in pregnancy there is a bound between maternal and neonatal metabolism, despite substantial qualitative and quantitative differences between the two types of urinary profiles. Since metabolomic is sophisticated technique and they use highly complex data interpretation tools, the correlation found between the two types of profiles is strong and clearly visible (Pinto J,et al. 2015). If every newborn is a reflection of her mother, there will be much wider margins of clinical and therapeutic action on which to act. A first step could be identifying multiple metabolic profiles associated with physiological pregnancies, in order to create a metabolomic database that can enclose indicative cluster of a pregnancy free of complications. It is important to investigate whether the strong correlation between the two types of metabolomics

profiles (mothers-newborns) can be quantified and highlighted from the very first stages of pregnancy, in order to foresee on eventual intrauterine abnormal development, even in early phases of pregnancy. Recently has been reported that maternal diet may influence offspring's health, even within well-nourished populations (Fotiou M, et al. 2018). The knowledge that the newborn metabolic profile reflects the flux of nutrients and other metabolites between the maternal and placental-foetal unit is crucial, paving the way for future studies to understand the effects of maternal biochemistry, physiology and lifestyle behaviours on foetal programming and infant outcomes. Walejko et al. provided information on the metabolic profiles of maternal and foetal placental tissues delivered by caesarean section showing that there are different metabolic alterations in the maternal and foetal tissues of the placenta following delivery (Walejko JM, et al. 2018). In the light of these results, our study on the "omics" appears paramount for a better understanding of the bond between the mother and the newborn. The indepth knowledge of the metabolism of each pregnancy is very important, not only for a good state of maternal health, but also because we could prevent or intervene, in advance, in situations in which foetal well-being is at risk. This would represent, in the clinical field, a screening tool, relatively low-cost and non-invasive, for some disorders in pregnancy involving pathophysiological alterations of the same metabolism.

5.2.5 Strengths and limitations

The current study was the first to utilize metabolomics, a technology that provides highly discriminating power and sensitivity, to investigate the comparison of the metabolomic profile of mother and newborn during normal full-term pregnancy. The study is limited in that we didn't know which were the metabolites that support the correlation

between the mother and her child. However, the information presented here reveals that each newborn is a mirror of the metabolic environment of the womb.

5.2.6 Conclusions

The metabolic profile of newborn correlates with the maternal one at 3 weeks before delivery, suggesting that the newborn is plausibly "programmed" by the maternal metabolism and this happens, most likely, even in earlier phases of pregnancy. Since the newborn metabolic profile reflects the flux of nutrients and other metabolites between the maternal and placental-foetal unit, metabolomic approach appears to be crucial to understand the effects of maternal biochemistry, physiology and lifestyle behaviours on foetal programming and infant outcomes.

5.3 "*Metabolomic profile of young adults born preterm"* (Published on Metabolites, 2021)

5.3.1 Introduction

Fetal and extrauterine life represents a continuum, during which the growth and development of the human being are influenced by genetic, environmental and social factors. Numerous studies have identified prematurity as a risk factor for the development of chronic adult diseases such as obesity, insulin resistance (Hofman PL,et al.2004; Tinnion R,et al.2014) and hypertension (Hack M, et al.2005; de Jong F,et al.2012). Recent evidence documents the fetal, rather than postnatal, origin of some chronic adult diseases. It is likely that fetal reprogramming occurs when the normal pattern of fetal development is disrupted by an abnormal stimulus or an "insult" during intrauterine life, which leads to adaptations by the fetus to allow for its survival, but could ultimately result in permanent structural and physiological changes with long-term consequences in adulthood. Early in utero life is vulnerable to perturbation, and compelling evidence indicates that the fetal period of development is extremely sensitive to environmental cues. Insufficient fetal substrates determine permanent structural and physiological changes, leading to long-lasting consequences in postnatal life (Barker DJ, et al.1995; Perrone S,et al.2016), Many experimental studies have been conducted to explain the phenotypic consequences of fetal–placental perturbations that predispose individuals to the genesis of metabolic syndrome in adulthood. Metabolomics is an emerging omics science, considered today as the key for personalized medicine, able to correlate the biochemical changes (characterizing the organism of the human being, exposed to multiple intrinsic and extrinsic stresses) with a determined phenotype, and obtaining real information about the state of health of a subject at that precise moment (Ellis DI,et al.2007). Metabolomics has already identified significant differences at birth in the profile of preterm newborns compared to those born at term. Gracie et al. studied the importance and value of omics technologies and integrated them precisely for the study of preterm newborns (Gracie S,et al.2011). Distinct metabolomic profiles were identified in infants born at different gestational ages, both in term and in preterm newborns (Atzori L, et al.2011) and in fetal growth-restricted infants (Dessì A, et al. 2011). However, very few studies have extended the follow-up of preterm infants into adult life (Thomas EL, et al.2011; Parkinson JRC, et al.2017; Atzori L, et al. J Matern Fetal Neonatal Med. 2011).

The aim of our work is to identify and to compare the metabolomic profile of young adults born preterm to term controls, testing the hypothesis that metabolic profile in adulthood differs according to gestational age and resembles that of birth.

5.3.2 Results

One hundred and twenty-eight preterm newborns met the inclusion criteria. Among them, 23 were deceased at the time of enrolment and 32 were untraceable through the available contact details. Nine were ineligible according to the exclusion criteria and 15 denied consent to participate in the study. Nineteen young adults born at term in the same study period (years 1990–1997) were selected as

controls. One of them denied consent while the study was underway (figure 11, corresponding to Figure 1 of the published article attached below as Annex 12).

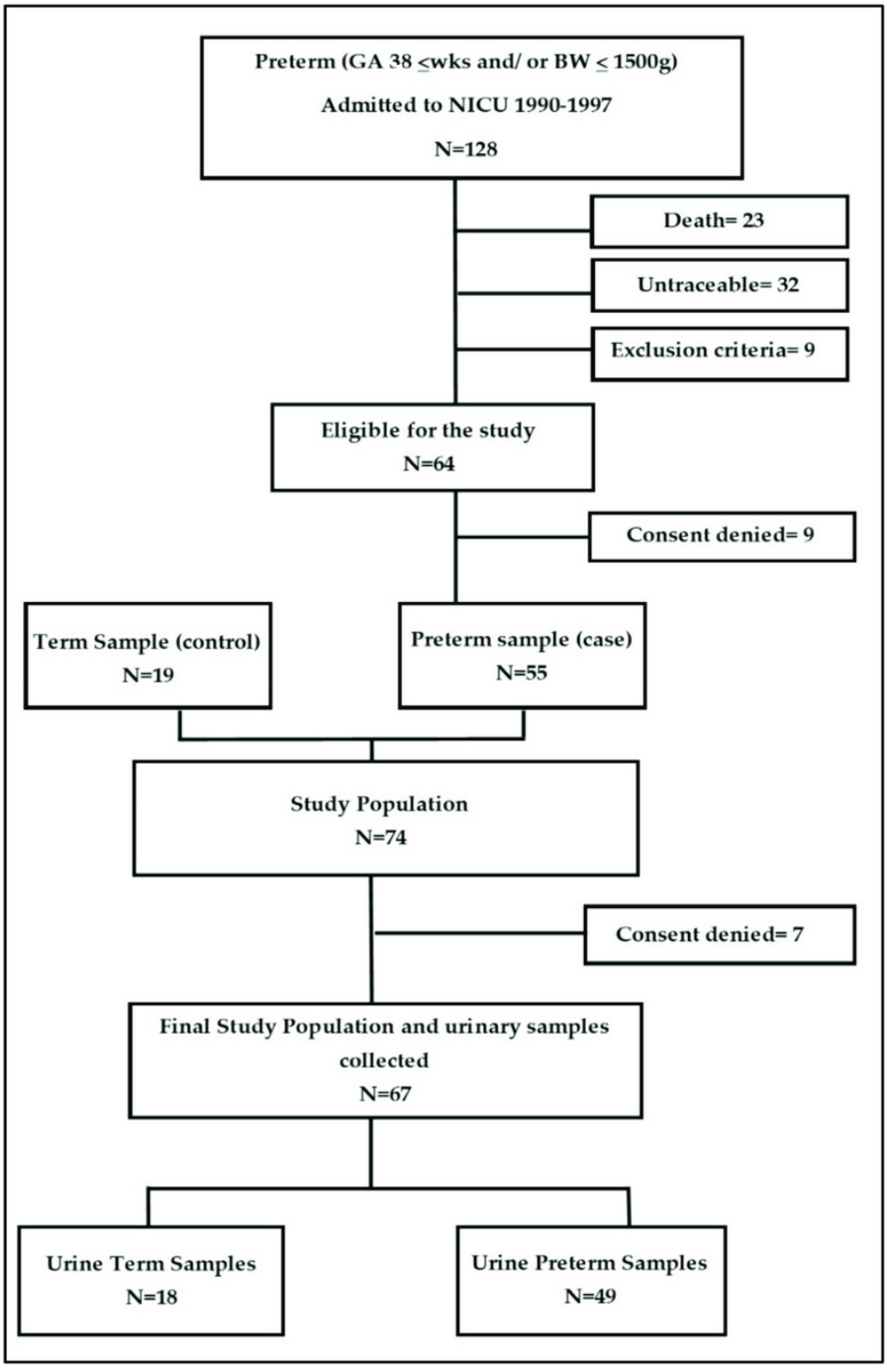

Figure 11. Participant flow chart; GA: gestational age; BW: body weight.

Therefore, the final study population consisted of 67 young adults: 49 born preterm (18 females and 31 males; gestational age: 30.25 ± 2.7 weeks; birth weight: 1131.91 ± 118.15, current age: 21 ± 2.4 years) and 18 born at term (6 females and 12 males; gestational age: 38.5 ± 1.4 weeks; birth weight: 3120.43 ± 261.02; current age: 20.9 ± 2.5). For the clinical characteristics of the enrolled population, see table 5 (Table 1 Annex 12).

Table 5. Perinatal and actual data in case and control groups.

Variables	Cases (n = 49)	Controls (n = 18)	p-Value
Maternal age (years), mean (SD)	31.19 (4.72)	31.15 (4.04)	Ns
Gestational age (weeks), mean (SD)	30.25 (2.72)	38.52 (1.44)	<0.05
Birth weight (grams), mean (SD)	1131.91 (118.15)	3120.43 (261.02)	<0.05
Male gender, n (%)	31 (63.26)	12 (66.6)	Ns
Apgar score at 1 min, median (IR)	5 (1–10)	9 (8–10)	<0.05
Apgar score at 5 min, median (IR)	8 (1–10)	10 (10–10)	<0.05
Neonatal resuscitation, n (%)	43 (87.7)	-	-
Intraventricular hemorrhage, n (%)	16 (32.6)	-	-
Hospital stay (months), mean (SD)	2.15 (1.11)	-	-
Age at assessment (years), mean (SD)	21.68 (2.42)	20.95 (2.55)	Ns
Caucasian population, n (%)	47 (95.9)	18 (100)	Ns
Same region of residency, n (%)	48 (97.9)	16 (88.8)	Ns
Actual mean systolic/diastolic blood pressure values (mmHg)	105/73	108/75	Ns
Actual body mass index < 18.5, n (%)	11 (22.4)	4 (22.2)	Ns
Actual body mass index 18.5–25, n (%)	34 (69.4)	13 (72)	Ns
Actual body mass index > 25, n (%)	4 (8.1)	1 (5.5)	Ns
Sport, n (%)	16 (32.6)	7 (38.9)	Ns

Ns: non significative.

Multivariate (chemometric) analysis allowed us to highlight differences in the urine metabolomic profile between young adults born preterm and young adults born at term. A non-supervised Principal Component Analysis (PCA) technique was performed to find clusters within the data set. The PCA did not show a clear difference between “preterm” and “term” clusters (figure 12, corresponding to Figure 2 of the pblished article).

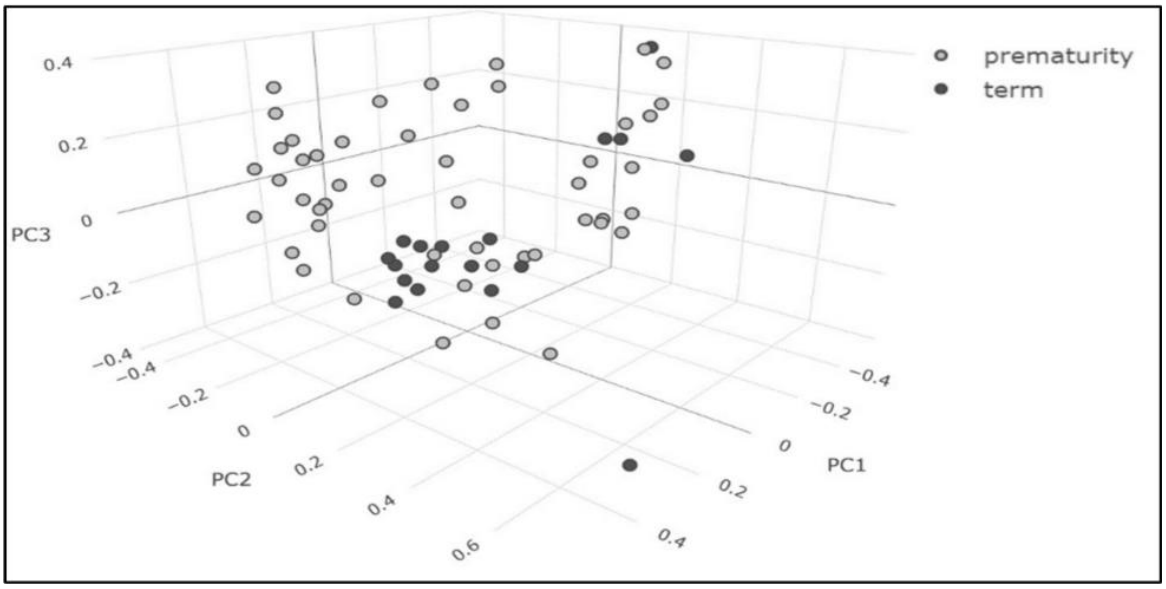

Figure 12. PCA score plot of the first three principal components; the two classes of patients, “preterm” and “term”, are represented in light gray and black points, respectively; PC: principal component.

Therefore, the next supervised step by means of a supervised technique was required. Firstly, classification tasks were performed using nuclear magnetic resonance spectral data as the input for the classification models. A leave-one-out cross-validation technique was used as a resampling method to estimate the models' performance. The models, nevertheless, were not yet very discriminative (the accuracy, i.e., the percentage of patients correctly classified by the predictive algorithm, was about 70%, see table 6 corresponding to Table 2 annex 12).

Table 6. Performance of the classification algorithms, obtained using scaled spectral data as input.

	Accuracy	F1 Measure	False Positive Rate	False Negative Rate	True Positive Rate	True Negative Rate
RF	0.7	0.82	0.94	0.06	0.94	0.06
GBM	0.72	0.82	0.72	0.12	0.88	0.28
SVM	0.73	0.84	1	0	1	0

RF: Random Forest; GBM: gradient boosting machine; SVM: support vector machine.

Therefore, classification tasks were performed using principal components as input data. The models' performances were estimated using 3 to 10 principal components to select the number of principal components with the best overall fit for each model (figure 13, corresponding to Figure 3 annex 12).

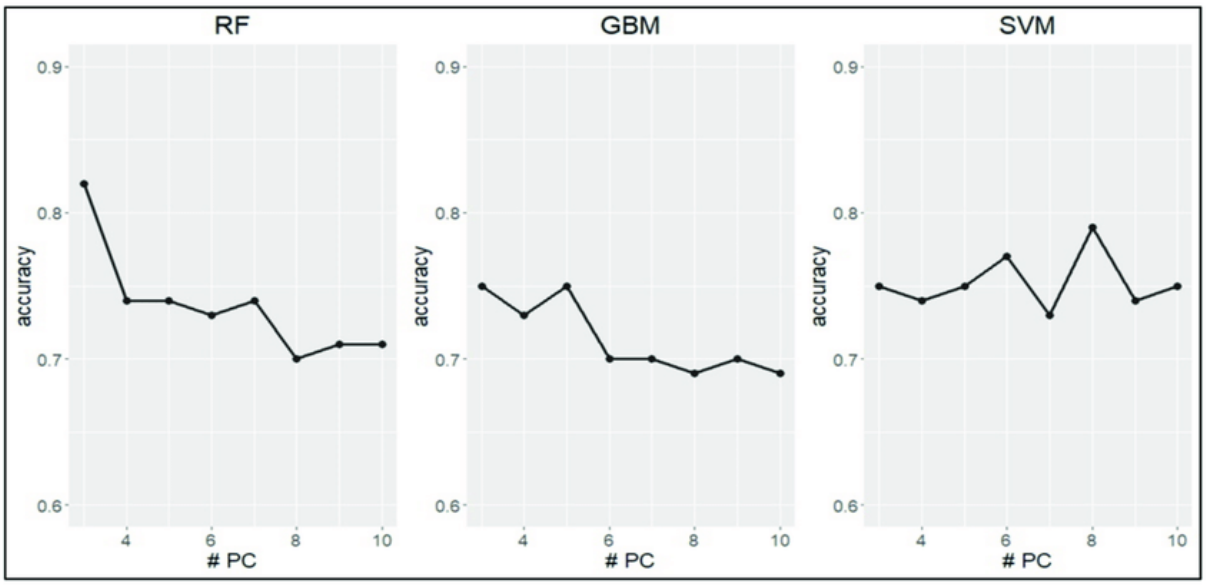

Figure 13. Accuracy of the models, estimated through the use of 3 to 10 PC as input data. RF: Random Forest; GBM: gradient boosting machine; SVM: support vector machine; PC: principal component.

The best classification result was obtained using the Random Forest (RF) model and the first three principal components as variables (accuracy ~82%). Moreover, to understand the contribution of each of the aforementioned components to the classification, an "importance" measure was computed through the RF algorithm. In order to identify the discriminating metabolites between preterm and term groups, the first three main components were then analyzed. The values of the loadings for each component were reported to understand how the metabolites contribute to each of the principal components (figure 14, corresponding to Figure 4 annex 12).

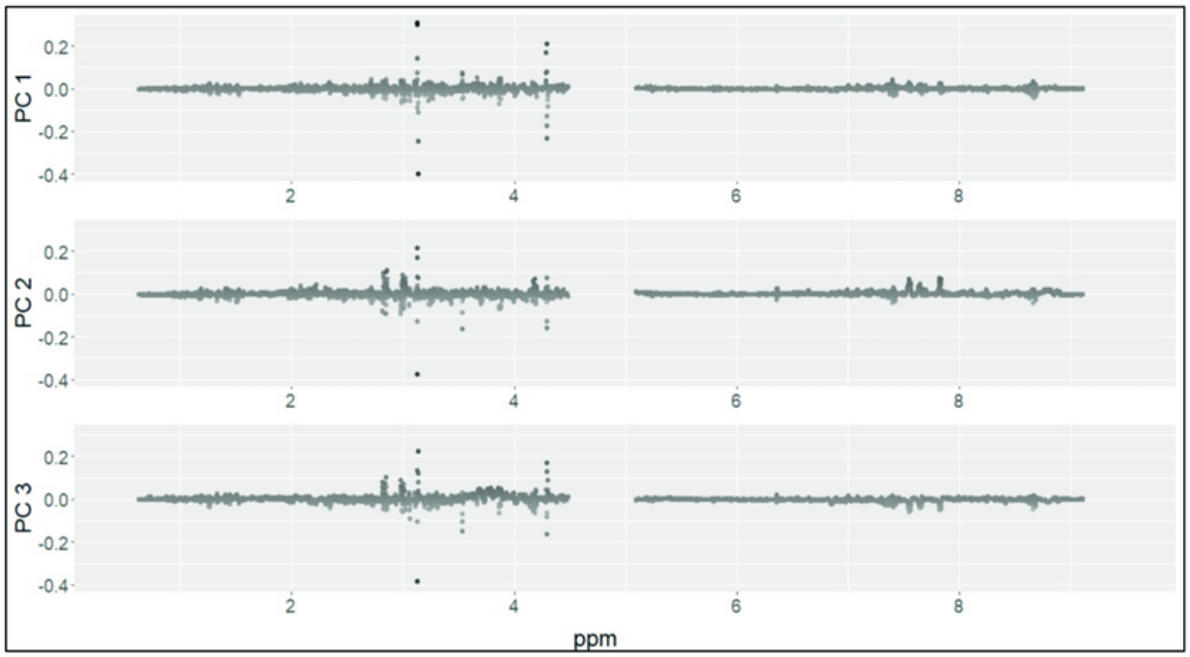

Figure 14. Values of the loadings of the first three principal components; the graphs illustrate which metabolite spectra (ppm) were most responsible for the "variance" in each of the three main components (i.e., the metabolite that has the higher absolute values).

Positive values of the loadings indicate that a variable and a principal component are positively correlated; negative values indicate a negative correlation. Large (either positive or negative) values of the loadings indicate that a variable has a strong effect on that principal component. Some thresholds were set to select the variables with the highest absolute loading values (figure 15, corresponding to Figure 5 annex 12).

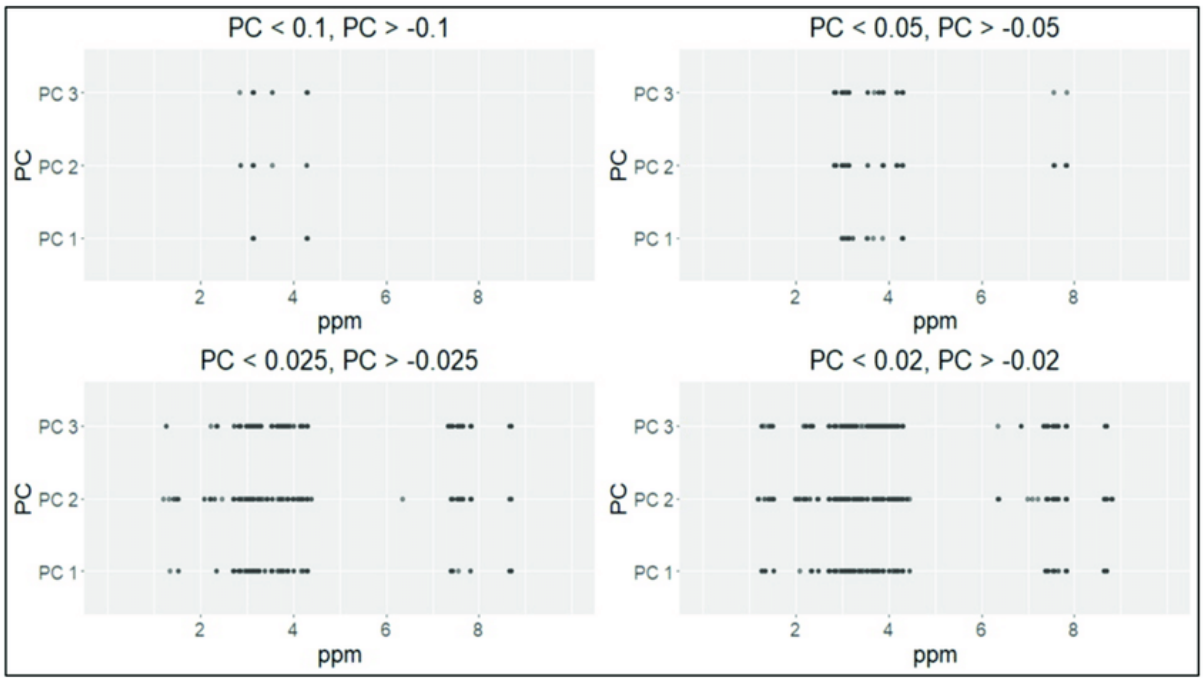

Figure 15. Different thresholds applied to the loadings of the first three principal components.

The threshold values 0.1 and 0.05 were too high (only a few metabolites were selected for these values). For the threshold values 0.025 and 0.02, four ranges were identified in the ^{1}H-NMR (proton nuclear magnetic resonance spectroscopy) spectrum: 1.3–1.5 ppm, 2.7–4.3 ppm, 7.4–7.8 ppm and 8.6–8.7 ppm (figures 16 and 17, corresponding to Figure 6 and Figure 7 annex 12).

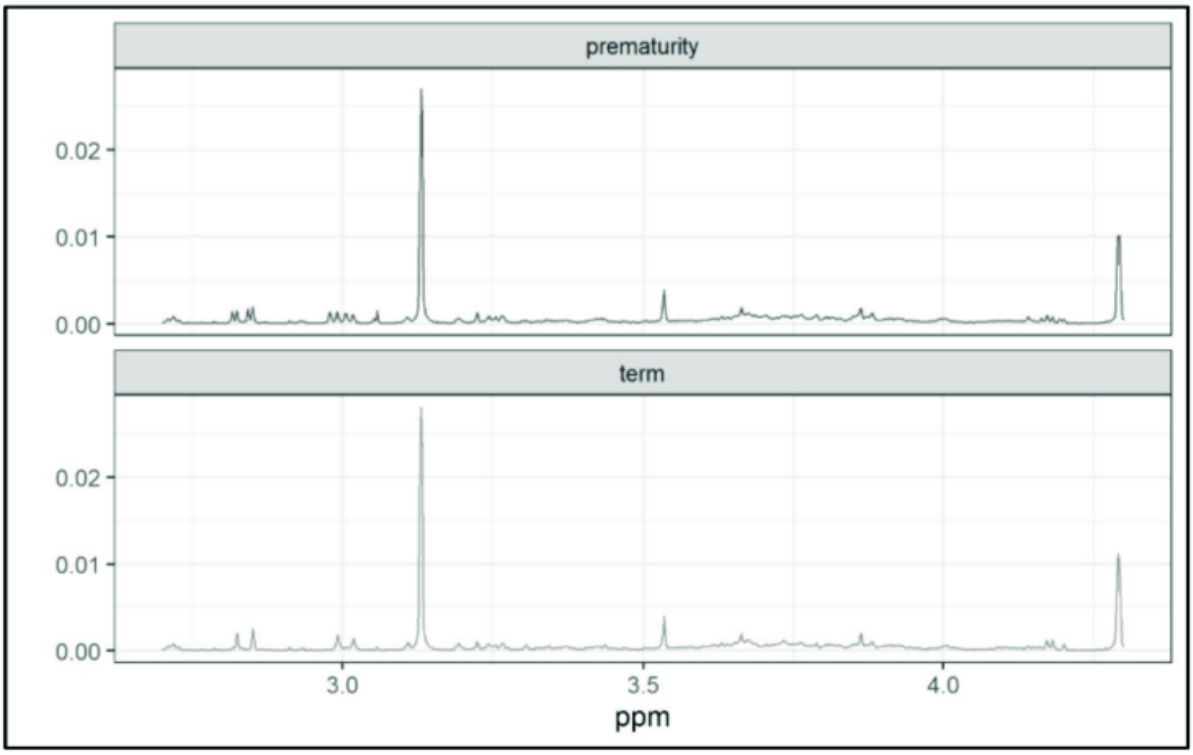

Figure 16. Comparison of mean spectra from each the two groups.

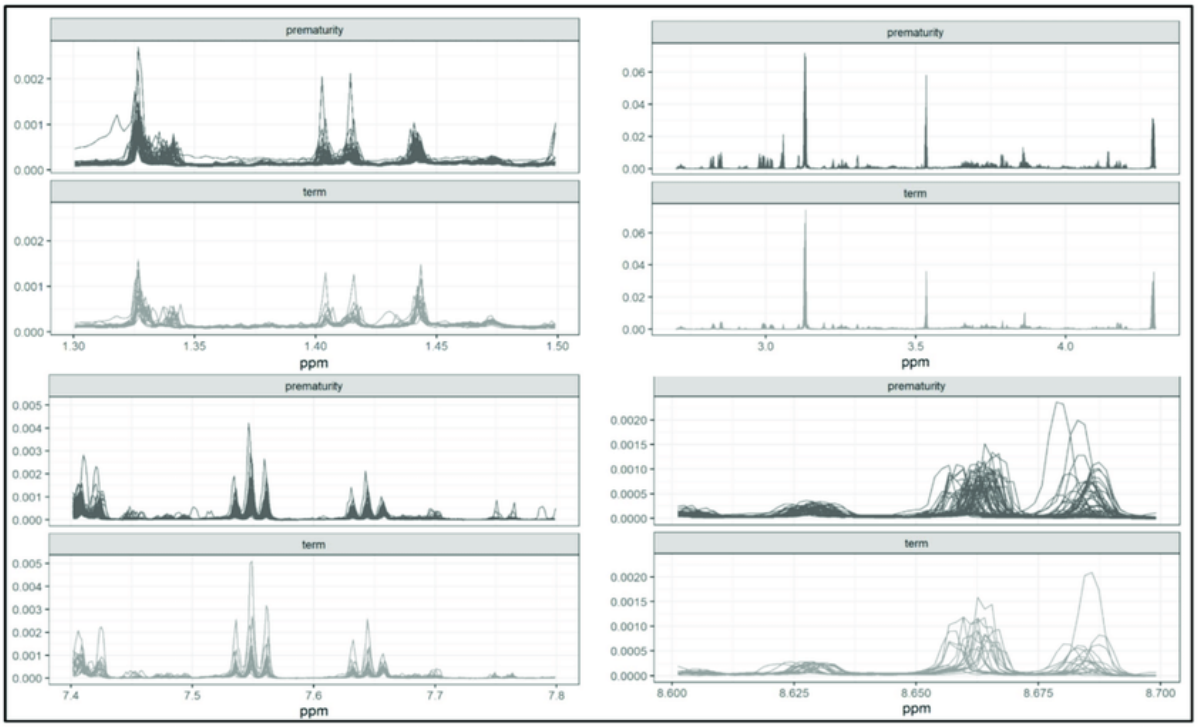

Figure 17. Spectra of the most important signals in the two groups.

The most significant signals, which distinguished the metabolome of preterm from that of term newborns, came from the following metabolites: citrate (3.13 ppm), CH2 creatinine (4.28 ppm), fumarate (6.8 ppm) and hippurate (7.6–7.8 ppm).

5.3.3 Discussion

The main finding of this research was that the urine metabolomic profile of adults born preterm significantly differed from the metabolic profile of adults born at term. With the unlabeled metabolomics approach, we were able to identify the significant spectra, which differentiated the two young adult populations (preterm vs. term). In particular, the involved metabolomic cycles most related to the characterizing metabolites found in the group of preterm (citrate, CH3 creatinine, CH2 creatinine, fumarate and hippurate) were tyrosine metabolism, tryptophan and phenylalanine biosynthesis, the urea cycle and arginine and proline metabolisms. Interestingly, these metabolomic patterns were the same as those found and described by Atzori L et al. in preterm newborns at birth, suggesting that the metabolomic profile of a young adult born preterm mirrors that of their perinatal period (Atzori L, et al. 2011). The same urine metabolites were also identified as influent in a recent study describing the variation in urine metabolites during the catch-up growth in the first months of life (Scalabre A, et al.2017). In this study, the Authors found that hippurate and other metabolites were related to an individual's weight, while citric acid and creatinine were both related to a subject's weight and height. In the case of citrate, which is part of several pathways regulating carbohydrate, fat and protein metabolism, age-dependent concentrations have been reported in other metabolomic studies. Creatinine is the waste product of the energy muscle metabolism; it is constantly excreted through glomerular filtration, and its concentration in urine and in blood is routinely used as a marker of renal function. Creatinine urinary level appeared to increase with increasing age and body weight, following the increase in muscle metabolism that occurs during childhood and early adult life, with an increase in physical activities (Scalabre A, et al.2017; Chiu CY, et al.2016). The finding that there are specific metabolomic patterns in young adults born preterm that mirror those found in the neonatal period and differ from those found in young adults born at term, confirm that biological samples have unique and distinctive biochemical compositions, which change in response to physiological (body weight, height and age) or pathophysiological stimuli (preterm birth). We hypothesized that an intrauterine environment that is not favorable for optimal embryonic and fetal growth may cause a placental and fetal "reprogramming" with changes in growth patterns and body metabolism that persist, unaltered, over the years (Dessì A, et al. 2013). Previous metabolomic studies performed in premature infants have already shown a difference in the levels of amino acids, enzymes and endocrinological markers collected from blood samples in the period immediately after birth (within 24–72 h from birth), showing that children at different stages of prematurity are metabolically distinct (Wilson K, et al. 2014). Moreover, it is already known that the adverse environment that preterm infants face during the preconceptual, fetal and postnatal period may have long-lasting effects on their adulthood health (Gluckman PD, Hanson MA. 2004; DiBattista A, Chakraborty P.2018). Therefore, the "snapshot" produced by the metabolomics

provides fingerprinting of the state of health, useful for investigating the body's metabolomic responses to the disease and external stimuli (Kim OY, et al. 2013). Although we believe in the relevance of the link among prenatal environment, fetal growth and adulthood health status in the predictive role of metabolomics in perinatology, the data are too limited to draw definite conclusions regarding the use of metabolomic profiles in clinical practice. Potential confounders (such as dietary intake and hormonal status) should be analyzed in detail and will benefit from studies on a larger number of patients to identify the effect of environmental factors and comorbidities on the metabolomics spectra. In our population, we did not find an association with hypertension or obesity, and we were not able to identify biomarkers for the risk of chronic disease in adulthood. This study has the limitation of including a small number of term control young adults and this may have affected the results for the personal profiles. However, the study population was well defined, with no variability in respect to location, lifestyle and eating habits. Gender and the related hormonal differences may also have influenced these results. This study therefore represents a preliminary phase, and a validation of our results in a new and larger cohort is necessary to check their reproducibility. Looking at the growing global incidence of chronic metabolic diseases, this research contributes to unveil the main routes of reciprocal linking between environmental factors and genetic susceptibility factors. Epigenetic modifications consequent to intrauterine environmental stimuli may persist long after the stimulus has ceased, providing a mechanism to explain the long-term consequences of acute exposures in early life. Metabolomics and ^{1}H-NMR allow the analysis of biofluids or tissues to extract latent information and enable sample classification and biomarker identification. Although plasma, serum, amniotic fluid, cord blood or stool can be used for metabolomic analysis, urine samples, due to their non-invasive method of collection, are a very promising tool in the pediatrics and neonatology field. The future goal will be to identify more accurately patients at risk for chronic adult diseases, for which an individual therapeutic approach might be necessary.

5.3.4 Materials and Methods

An observational case–control monocentric study was carried out at the University Hospital of Siena, in the Neonatology–Pediatrics Unit. The urinary samples were collected from young adults recruited in the research study, "Multidisciplinary long-term follow-up of premature births: AOUS case series 1990–1997". They were enrolled to take part in the multidisciplinary follow-up study that was conducted at the University Hospital of Siena.

Inclusion and Exclusion Criteria

The study population was enrolled starting from a cohort of young adults born with gestational age (GA) ≤ 33 weeks and/or birth weight ≤ 1500 g, admitted to the Neonatal Intensive Care Unit at the Santa Maria alle Scotte Hospital, in the period between 1 January 1995 and 31 December 1997. Babies born at term in the same study period (years 1995–1997) were selected as controls (for the clinical characteristics of the enrolled population, see table 5 above and Table 1 Annex 12 below). Subjects suffering from genetic or malformative syndromes, inborn errors of metabolism, severe motor disability

and all whose conditions prevented the completion of the performance-expected tests, were excluded from the study. Vegan or vegetarian diet and alcohol use also represented exclusion criteria. The study was conducted in accordance with the ethical principles enshrined in the Helsinki Declaration's latest revision. Patients eligible for the study were contacted by telephone and informed about the aims and methods of carrying out the study. Adherence to the study was voluntary. Nevertheless, official participation in the study was subject to the signing of an informed written consent, which guaranteed all rights regarding the protection of personal data according to the national law.

Clinical Data Collection

Eligible patients were invited to the Neonatology–Pediatrics Unit, Neurodevelopmental Follow-up Division. For each patient, we drew up a clinical folder, consisting of: a signed copy of the informed consent; data relating to the perinatal age retrospectively collected from the birth medical records (such as gestational age, birth weight, type of delivery, length of hospital stay at birth, complications or problems related to prematurity that came out during hospitalization and diagnosis at discharge); data related to the current state of health of the patient; the anthropometric parameters (including height, weight and body mass index achieved); and the clinical examination.

^{1}H-NMR

Urine samples were collected and shipped in dry ice to the Laboratory of the University of Siena. The samples were then analyzed using the ^{1}H-NMR analysis technique. ^{1}H-NMR measurements were performed on a Bruker DRX600 MHz Avance Spectrometer with a selective inverse probe equipped with a Z-gradient coil, as previously described (Perrone S, et al.2020). Briefly, spectra were acquired at a constant temperature of 298.0 ± 0.1 K using a 90° pulse. A delay of 10 s was included in the pulse sequence to obtain the relaxation time T1. In fact, the values of T1 (in the range 1.5–2.8 s) of the considered metabolites were such that a delay of 10 s allowed for the full recovery of the longitudinal magnetization after a 90° pulse, as verified by integral values constant for D1 ≥ 5 s. A saturation pulse of 2 s suppressed the water signal during the water resonance. A total of 32 k data points per scan were used and 128 transients were accumulated. Each urine sample was measured after centrifugation occurred, 2000 ppm for 5 min. The pH of the urine samples was checked with a buffer solution (pH 7.4) containing trimethylsilylpropanoic acid (TSP). Samples (550 µL) plus 50 µL of TSP-d4 20 mM solution were measured into the 0.5 mm tube (tube diameter) of the ^{1}H-NMR. All ^{1}H-NMR spectra were first performed at their physiological pH. This first spectrum was used only to obtain an overview of the metabolites contained. A second spectrum was executed at pH 1.0 ± 0.02 in the same MR tube, with a microelectrode. The chemical shift of ionizable fluids is highly dependent on the pH. At a pH of 1.0, all chemical shift values were reproducible within ± 0.01 ppm. Spectra were aligned to compensate for the shift of the signals of some metabolites, due to small inter-sample pH changes. Then, they were uniformly binned to 0.0025 ppm intervals between 0.5 and 9.5 ppm, excluding the region corresponding to water (4.6–5.2 ppm) and TSP (−0.5–0.5 ppm) signals. Bins were normalized to the total spectral area to compensate for the different dilutions of original urine samples. To identify the most discriminating

parts of the spectrum, the results of the classification algorithm were combined with the profiles of the respective loadings. A system of thresholds, defined empirically, then allowed the selection of the characteristic parts of the spectrum of the two groups.

Statistical Analysis

The data were analyzed using the R program (R Core Team (2016). R: a language and environment for statistical computing R Foundation for Statistical Computing, Vienna, Austria. Available online: **https://www.r-project.org** (accessed on 5 October 2021)). The data of sample characteristics with a normal distribution were evaluated by unpaired t-student test, while categorical data were analyzed by chi-square test. The study was conducted according to the classical metabolomic approach divided into two steps: an unsupervised and a supervised phase (Tataranno ML, et al. 2018). In order to find clustering evidence, a non-supervised technique (PCA) was performed on mean-centered and Pareto-scaled methods data. PCA was also used to detect possible outliers within the dataset. The next supervised step allowed us to model data through different classification systems: RF, support vector machine and gradient boosting machine. These different machine-learning algorithms were used to analyze the differences in the metabolomic profile that were connected to the different gestational age at birth (Lee J, et al. 2021; Menin D, et al. 2019; Sufriyana H, et al, 2020). As the classification algorithms employed do not provide direct methods for calculating the significance of the variables responsible for classification, alternative methods were used to define which elements could support the performance of the model. To select and identify metabolites that distinguish young adults born preterm from those born at term, a threshold method was used. By varying the threshold of interest, it is possible to look for the metabolites best expressed by the various principal components, and to estimate which are the most important for defining the classification. A threshold method allowed us to combine the rigor of a systematic approach (choice of classification model and identification of the most important principal component) with a more manual approach to test and choose the selection thresholds. It also allowed us to see if metabolites emerge, establishing a significance of the effects.

5.3.5 Conclusions

Urinary spectra were able to discriminate the metabolomic profiles of young adults born preterm from those born at term, revealing differences similar to those already reported at birth. Urine spectra may provide insight into the peculiar metabolomics of preterm babies that persists into adulthood, paving the way for further research on the pathogenesis and effects of fetal programming on infants' outcomes. This work is preliminary research that opens the interest of neonatologists to the fingerprinting of prematurity. In-depth knowledge of the metabolomics of preterm babies is very important, not only for a good state of childhood health, but also because we could prevent or intervene, in advance, in situations in which neonatal development is at risk to become poor. This would represent, in the clinical setting, a relatively inexpensive and non-invasive screening tool for some early-life pathologies that involve pathophysiological alterations of the metabolism itself.

CHAPTER 6

CONCLUSIONS AND FUTURE PERSPECTIVES

Parallel to the denatality and the drastic decline in physiological births in the Italian reality, we are witnessing a progressive increase in the number of infants at risk, potentially fragile subjects until adulthood: this is linked both to the increase in high risk infants and children (such as VPTs) thanks to the improvement of clinical care and survival, but also to the numerical increase of those infants at lower risk (such as MPTs and LPTs), with an overall increase in the long term of economic and social costs, especially for the management of the neurodevelopmental and chronic metabolic aspects.

The critical review of the literature conducted in recent years has led to confirm the role of oxidative stress in the physiopathology of the conditions characterizing the fetus and newborn at risk. Oxidative stress is indeed a critical factor for fetal programming, representing a key process that correlates inadequate fetal growth, impaired fetal well-being or preterm birth with the subsequent increased risk of disease in adolescence and adulthood (Buonocore G, et al. 2017). However, currently the analysis of OS biomarkers that would have a possible diagnostic, monitoring, predictive, prognostic and pharmacodynamic role, is still confined to the field of experimental and clinical research but not usable in clinical practice, due to the complexity of the procedural techniques, the lack of automation and the high cost that have hindered their routine use in the clinical setting: overcoming these technical and economic difficulties represents a challenge for the immediate future, since an accurate assessment of oxidative stress would contribute to improve the quality of neonatal care (Torres-Cuevas I, et al. 2017), offering the possibility to refine early therapeutic strategies.

Currently there are still few antioxidants used and approved, especially in the neonatal setting, considering the therapeutic potential offered by this class of compounds and drugs. To counteract free radical damage, strategies in preclinical and clinical studies have tried to increase the antioxidant status of full-term and preterm infants, and melatonin, which is a safe, non-toxic and effective molecule, plays a leading role, being tested against “oxygen-free radical diseases” of the newborn with promising results (Tarocco A, et al. 2019), also confirmed by our study.

We have also noted that the current challenge of medicine, including neonatal medicine, is represented by the development of personalized care solutions according to a tailored approach, i.e. tailored to the individual patient, especially in the case of small critical or fragile patients such as newborns at risk, and this in turn justifies the growing need for a critical analysis of perinatal data aimed at the “benchmarking” process. In neonatology there is not only the newborn, but the mother-fetus dyad and the mother-newborn dyad or more precisely the mother-placenta-fetus/newborn triad, in a complex framework of reciprocal interactions that contribute to programming and developmental reprogramming, that is the response of the developing organism to a specific challenge during a critical time window that qualitatively and/or quantitatively alters the trajectory of development with consequent persistent effects on the phenotype (Fanos V, Yurdakök M.2010).

Even through our metabolomics works, albeit preliminary, we have shown that mother and child represent a reciprocal mirror as regards the metabolic profile, and this evidence underlines the importance of preventive or early interventions, from the very early stages of pregnancy, on subsequent neonatal outcomes and above all in the long term: what Barker hypothesized (Barker DJ. 1990), therefore finds a progressive biological and molecular confirmation through the metabolomic approach. The finding that specific metabolic molecules exist in young adults born preterm and that mirror those found in the neonatal period but differ from those seen in young adults born full-term confirms that biological samples have unique and distinctive biochemical compositions that are variable and modifiable in response to physiological or physiopathological stimuli (such as preterm birth). The intrauterine background, more or less physiological or pathological, and the prematurity itself seem to leave a long-term trace, conditioning the development of a metabolic pattern or profile that subjects carry around throughout their life like a fingerprint; however, further confirmatory studies are needed, as our conclusion is based on comparison with literature data and long-term longitudinal metabolomic studies would therefore be necessary to verify and confirm the hypothesis on the same subjects.

Further studies also seem necessary to verify the differences between prenatal metabolic profiles, those at birth and in adulthood, especially in the comparison between subjects with similar clinical history but different long-term outcomes, in addition to the similarities: the clinical relevance of these differences would be correlated to the influence of postnatal factors (environmental, epigenetic) in subsequently modifying the distinctive metabolic pattern of the individual subject, and this would also open the way to search for later therapeutic opportunities. Overall, therefore, the growing speed of knowledge in biomolecular research together with the rapid development of high-tech diagnostic systems has significantly contributed to the increase in neonatal life expectancy, and is increasingly contributing to the increase and improvement of a "personalized" assistance; however, new efforts are needed to narrow the growing gap between translational research and clinical practice (Mussap M, et al. 2013). Indeed, the link between prenatal environment, fetal growth and health status in adulthood and the predictive role of metabolomics in peri-neonatology appear increasingly evident, but the data are still too limited to draw definitive conclusions on the use of the metabolomic profile in clinical practice.

The so-called '-omics' sciences have been formulated to define approaches capable of identifying groups of biomarkers characteristic of a particular disease, possibly with preventive and predictive purposes; among these sciences, metabolomics may have the extraordinary ability to identify a clinically significant panel of metabolites in acute or chronic neonatal clinical conditions (Fanos V, Yurdakök M.2010; Mussap M, et al. 2013), with the advantage of relatively low costs and non-invasiveness.

In light of this, and on the basis of the critical review of the literature and preliminary studies conducted in recent years, future personal research perspectives aim to include the validation for the clinical use of the oxidative stress biomarkers studied in the peri-neonatal field; the development of a panel of oxidative stress biomarkers usable through the metabolomic approach with consequent cost reduction; the development of a panel of metabolic or better metabolomic biomarkers that can identify -for preventive,

predictive and prognostic purposes- the main prematurity-related diseases in the short- and long term, hopefully through prospective longitudinal studies. It is also desirable to evaluate and validate risk biomarkers even in newborns apparently at lower clinical risk but numerically more significant, such as MLPTs.

CHAPTER 7

SUMMARY OF THE THESIS

The improvement of peri-neonatal care has allowed a progressive drastic increase in the survival of high-risk preterm infants, i.e., very preterm or very low birth weight (VLBW) and extremely preterm or extremely low birth weight (ELBW) infants. However, the increased survival correlates with an almost stable incidence of disability as a result of the same prematurity, in particular for the lower gestational age (GA) groups. Furthermore, even infants born moderate and late preterm (32-36 weeks GA), although at a lower risk, are not exempt from clinical problems which also result in significant economic and social costs, considering the large number of these patients. In order to improve the short and long-term outcomes of these infants at risk, increasing attention in neonatology is focused on understanding the pathophysiological mechanisms underlying the prematurity-related diseases, and -at the same time- on the study of the individual diversification of these mechanisms. The concepts of *fetal programming* and *developmental reprogramming* represent the biological substrates that explain the importance of the mother-placenta-fetus/newborn triad in the realization of the long-term global health status and justify the interest in the development of a "precision neonatology", that is, of personalized care solutions according to a tailored approach. The identification of the newborn at risk and its potential problems therefore represents a fundamental step for the implementation of follow-up programs for these newborns, and vice versa an adequate follow-up path allows, over time, the critical analysis of the pathophysiological mechanisms and the perinatal data feedback: both of them represent essential steps in the benchmarking process, which in turn contributes to the definition of individualized strategies for improving clinical and care performance.

Throughout the diagnostic-care process that includes the identification of the newborn at risk, the individual risk stratification, the early diagnosis of pathology and the effects of any therapeutic intervention, biomarkers represent essential tools, such as those of oxidative stress (OS, that is critical for *fetal programming* and common denominator of many prematurity-related pathologies, called in fact "*free radical related diseases of prematurity*") and the potential biomarkers identifiable through the modern approaches of metabolomics, "the new clinical chemistry" [Antonucci R. 2010].

With these objectives, the Ph.D. research project has therefore been articulated on various preliminary work fronts, which can open the way to research aimed at developing a *precision neonatology* in a continuous evolution. The present thesis aims to summarize and unify the evidence-based scientific knowledge extrapolated from the literature and that obtained through the personal studies carried out, especially in the more recent field of metabolomics. The following topics are therefore addressed and illustrated: a brief introduction on the evolution in neonatology and the role and importance of biomarkers between research and clinical practice (Chapter 1); the conditions that define neonatal risk, even those less known and in which the long-term risk is less striking but significantly impacts on social and health costs (Chapter 2); the critical review of the literature regarding biomarkers of oxidative stress,

potential clinical biomarkers of diagnostic-prognostic utility in the preterm infant (Chapter 3); the possible preventive and antioxidant defense strategies in the newborn and the potential role of melatonin in preterm infants (Chapter 4); the application of metabolomics in neonatology between physiology and pathophysiology in the long-term follow-up of both full-term and preterm newborns (Chapter 5); finally, the conclusions and future perspectives of the research theme are briefly discussed, for a possible extension of the preliminary works presented through the project of this Ph.D. (Chapter 6).

In particular, regarding the definition of risk levels of the newborn, the literature data and personal contributions are illustrated through some clinical studies, to support the evidence of short and long-term problems both in very preterm ((“*Valutazione della densità minerale ossea in un gruppo di giovani adulti nati pretermine: il follow-up multidisciplinare.*” [evaluation of bone mineral density in a group of young adults born preterm: the multidisciplinary follow-up]; “*Personality, emotional and cognitive functions in young adults born preterm*.”) and in the moderate and late preterm infants (“*The moderate and the late preterm infant: comparison on neonatal outcomes.*”; “*The auxological outcome in the first year of life of the moderate and late preterm infants.*”). Underlying many diseases of prematurity, a determining role of oxidative stress (OS), the imbalance between the production of free radicals and antioxidant defenses to which the newborn -in particular preterm- is particularly susceptible, is now demonstrated; for this reason, special consideration is reserved to the critical review of the scientific literature regarding OS biomarkers, potential tools for the identification of newborns at risk, the early diagnosis of pathologies of prematurity and potential long-term health outcomes (“*Biomarkers of oxidative stress in the fetus and in the newborn.*”; “*Oxidative stress biomarkers in the perinatal period: diagnostic and prognostic value.*”). OS biomarkers are also useful tools to evaluate the effect of potential antioxidant agents under study, including for example melatonin: through a personal contribution represented by a double-blind randomized controlled pilot study, the early enteral administration of melatonin in preterm newborns has shown to be an effective strategy in reducing lipid peroxidation and therefore the OS levels in these infants at risk (“*Antioxidant effect of melatonin in preterm newborns*.”). Finally, part of the research project is dedicated to the innovative discipline of metabolomics, the most recent of the omics sciences and probably the one of greatest interest and applicability in the peri-neonatal field: providing a snapshot of the metabolic state, similar to a fingerprint of the individual, the biomarkers obtainable through metabolomic studies represent potentially useful tools to describe physiological and pathophysiological variations that contribute to the onset of pathologies and diseases even in the long term. Considering the clinical relevance of the mother-placenta-fetus/neonate triad in *fetal programming*, we first used a metabolomic approach to investigate the comparison between mothers and their newborns profiles during physiological full-term pregnancies, helping to demonstrate that the neonatal metabolic pattern mirrors that of the pregnant mother ("*Newborn metabolomic profile mirrors that of mother in pregnancy*."). Finally, through a case-control study as part of the long-term multidisciplinary follow-up project of preterm babies, the urinary metabolic profiles of young adults born preterm were compared with those of controls born at term, demonstrating significant differences

between the two groups (in relation to the following metabolites: citrate, creatinine CH2, fumarate, hippurate), and so confirming the importance of both the fetal programming on the future state of health, and of metabolomics as a potentially optimal tool to provide an individual snapshot of the state of health ("*Metabolomic profile of young adults born preterm*.").

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ANNEXES 1-12

ANNEX 1

Laschi E., Perrone S., Lembo C., Buonocore G. *Neonatology: Evolution from the Past to Future Perspectives.* Journal of the Siena Academy of Sciences; 10(1). https://doi.org/10.4081/jsas.2018.8528.

ANNEX 2

Certificate of participation as speaker to the congress of the Clinical Biochemistry Study Group of Italian Society of Neonatology (SIN) "Novità dalla ricerca in biochimica clinica neonatale". Firenze, 16 Settembre 2020.
Laschi Elisa. "*Il ruolo dei biomarcatori nella pratica clinica neonatale: dal laboratorio al letto del malato*".

ANNEX 3

Serafina Perrone, Elisa Laschi, Elisabetta Grande, Giuseppe Buonocore. *Bronchopulmonary dysplasia and oxidative stress in the newborn.* In: "Oxidative stress in lung disease (Vol-1)". Eds: Dr. Sajal Chakraborti, Dr. Tapati Chakraborti, Dr. Salil Kumar Das and Dr. Dhrubajyoti Chattopadhyay. Springer Nature Singapore Pte Ltd.2019. (https://doi.org/10.1007/978-981-13-8413-4_16. Published on 07/09/2019).

ANNEX 4

C. Petrolini, E. Laschi, S. Negro, F. Marinelli, S. Orlando, M. Giordano, S. Perrone. *Valutazione della densità minerale ossea in un gruppo di giovani adulti nati pretermine: il follow-up multisciplinare.* Abstract, XXVII Congresso Nazionale della Società Italiana di Neonatologia (SIN). 6-9 Ottobre 2021.

ANNEX 5

Laschi E., Nanni G., Giordano M., Muraca M.C., Palombo D., Buonocore G., Perrone S. *The moderate and the late preterm infant: comparison on neonatal outcomes.* 3rd jENS - Congress of joint European Neonatal Societies, Maastricht 17-21 September 2019 (poster).

ANNEX 6

Laschi E., Nanni G., Giordano M., Muraca M.C., Palombo D., Buonocore G.,Perrone S. *The auxological outcome in the first year of life of the moderate and late preterm infants.* 3rd jENS- Congress of joint European Neonatal Societies, Maastricht 17-21 September 2019 (poster).

ANNEX 7

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ANNEX 8

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ANNEX 9

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ANNEX 10

Lucia Marseglia, Eloisa Gitto, Elisa Laschi, Maurizio Giordano, Carmelo Romeo, Laura Cannavò, Anna Laura Toni, Giuseppe Buonocore, Serafina Perrone. *"Antioxidant effect of melatonin in preterm newborns"*. Published on Oxidative Medicine and Cellular Longevity (2021); 2021:6308255.

ANNEX 11

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ANNEX 12

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NEONATOLOGY: EVOLUTION FROM THE PAST TO FUTURE PERSPECTIVES

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Abstract. The begin of modern neonatology takes place in the 1940s, when physicians first started to have interest in the newborn so that the primary responsibility for the neonate passed from the obstetricians to the neonatologists. In the 19th century the term *premature* grouped together the concept of "preterm and weak infants", meant as babies suffering from poor energy and vitality. The idea that premature infants could be treated was introduced in the second half of the 19th century, when crucial fields signed the basis for neonatal care over the last century, such as thermoregulation, Apgar score, respiratory support, prenatal corticosteroids, metabolic screening and jaundice. From then on, advances in neonatology have resulted in the reduction of infant mortality worldwide. To date, scientific evidences have shown that the environmental conditions experienced in early life can profoundly influence human biology and long-term health. Chemical contaminants in water and diet, tobacco smoke, air pollution, gestational diabetes, hypertension and pre-eclampsia are all conditions that lead to the lowest common denominator *oxidative stress.* Fetuses and newborns -especially preterm- are particularly susceptible to oxidative stress mediated damage. Recently, the "*omics*" sciences represent the major area of growing interest and research in neonatology. The analysis of the metabolic profile detectable in a human biological fluid allows to instantly identifying changes in the composition of endogenous and exogenous metabolites caused by the interaction between specific physiopathological states, gene expression, and environment. From metabolomics studies comes the need of *individualized and tailored medicine.*

Key words: Newborn infant, Preterm newborn, Perinatal care, Metabolomics, Oxidative stress.

SCIENTIFIC ACHIEVEMENTS FROM THE PAST

In Biblical times, women were assisted by other women to stand on two bricks that were placed underneath their feet; the stones were dubbed "birthing bricks" and helped the midwife or assistant to have a little extra room to catch the baby. In the middle age, exclusively midwives assisted the births because men generally considered it uncouth to set foot in the delivery room. Of course this included the doctors, since they were men.[1] The best-selling midwifery handbook of the time "Rose Garden for Pregnant Women and Midwives", came out in 1513, offering advice on such things as removing a stillborn. Sitting on a birthing stool – basically a horseshoe-shaped wood chair without a seat – was advised for delivery.[2] In the 17th Century, 3 to 5 women shared a bed at the famous Paris hospital Hotel Dieu: "*if a woman died in childbirth, the other ones waited hours or even overnight for the orderly to cart the corpse away*," Epstein writes in Get Me Out.[3] The begin of modern neonatology takes place in the 1940s, when physicians first started to have interest in the newborn so that the primary responsibility for the neonate passed from the obstetricians to the neonatologists.[4] In the 19th century the term "premature" grouped together the concept of "preterm and weak infants", meant as babies suffering from poor energy and vitality. Many physicians had the idea that premature birth was the way of nature of expelling a defective fetus.[5] Almost always newborns were born at home, lying-in hospitals were only used if the mother was destitute. Over the course of the 19th century obstetricians focus their attention on the mother and rarely on the infant beyond initial resuscitation.[6] The idea that premature infants could be treated was probably introduced in the second half of the 19th century. The term *neonatology* was used for the first time in 1960 in the introduction of the first edition of the book *Diseases of the Newborn* by Alexander Schaffer.[7] From then on, there have been crucial fields that signed the basis for neonatal care over the last century, such as thermoregulation, Apgar score, respiratory support, prenatal corticosteroids, metabolic screening and jaundice.

Thermoregulation

At the beginning of the 20th century, neonatal mortality was high and the introduction of temperature regulation considerably reduced it. The invention of the incubator is associated with Stephane Tarnier, a French obstetrician who first tried to introduce a mean to contrast hypothermia that occurred routinely in numerous

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premature infants who succumbed on the wards of Paris' Maternitè Hospital in the 1870s. He statistically demonstrated the significance of this device comparing premature infant mortality before and after the use of it. Results were remarkable, in 500 infants in the 1200 to 2000 g range mortality dropped from 66% to 38%[8] A lot of types of incubators were conceived and used in Europe and the United States, until Silverman, Day *et al.* in the 1950s at Columbia, New York, revealed the benefits of controlling body temperature in term of reducing metabolic rate, oxygen consumption and hypoglycemia. Today's incubators also have the function to protect premature babies from infection, allergens, excessive noise and light levels that can be detrimental. In addition, incubators have now been developed as a multifunctioning system that comprises several tasks as the supplementation of oxygen, provision of nutrition, monitoring of vital parameters, administration of medications and maintaining fluid balance.[9]

Apgar score

In the early 1950s, an obstetric anesthesiologist, Virginia Apgar, developed a reliable assessment of the newborn at birth. Apgar's system of evaluation was then presented in 1952 at the 27th annual Congress of Anesthetists and International College of Anesthetists and published in Anesthesia and Analgesia in 1953.[10] Her system implies to evaluate the newborn using five parameters - heart rate, reflex activity, respiration, tone and color – within the first minute and reevaluation at 5 or, if necessary, 10 minutes.[11] This method remains still today a valid tool in neonatal assessment around the world.[12]

Respiratory support

From the late 19th century to 1950's ventilation was delivered to the body of the patient using sub-atmospheric pressure with the aim to replace or enhance respiratory muscles work.[13] In 1950 Allan P. Bloxsom introduced a new device, the positive oxygen air lock, to resuscitate and oxygenate the asphyxiated newborn in the delivery room. This unit provided warmed humidified 60% oxygen. The naked newborn entire body was placed in the pressure lock if "failure to breathe or breathe properly" occurred. The positive pressure was cycled between zero and five pounds per square inch to replicate the intrauterine pressures during the second stage of labor. The infant remained in the pressure lock until respirations became established and the infant well oxygenated. This treatment led him to the conclusions that the most important mechanism for the initial onset of respiration was absorption of oxygen through the skin and upper respiratory tract and that oxygen plays a fundamental role for the prompt stimulation of the respiratory center. The randomized, controlled clinical trial of the positive pressure air lock was finally published in 1956.[14,15] First attempts to provide continuous respiratory support through mechanical ventilation to infants with severe respiratory disease were made in the mid-1960s. At that age, most preterm infants died for respiratory distress syndrome (RDS) or hyaline membrane disease (HMD), as it was originally called. Since that was the apparent cause of death of infant son of President John F. Kennedy, research had been implemented to investigate the cause and management of the disorder. In 1959, Mary Ellen Avery and Jere Mead linked the pathogenesis of this disorder with the deficiency of surfactant in the lung.[16] Over the past 60 years, new modes of ventilation have been developed and many technical aspects improved such as flow delivery, use of microprocessors and exhalation valves. In the 1990's controlled ventilation moved towards partial ventilation support and finally pressure support ventilation.[13]

Prenatal corticosteroids

The discovery of therapy for fetal maturation happened upon incidentally, when the obstetrician Graham Liggins was studying in a sheep model factors involved in the initiation of labor, testing his hypothesis that steroid hormones might trigger labor. He found more mature lungs in preterm lambs exposed to corticosteroids in utero, that also were surviving at earlier gestational age and had milder respiratory distress.[17] Then Liggins and Howie conducted a randomized controlled trial of maternal administration of betamethasone, showing a reduction of incidence of RDS in preterm infants and neonatal mortality.[18] In 1990, a meta-analysis published by Crowley and colleagues showed that antenatal betamethasone has a protective effect against the development of also other neonatal morbidities such as intra- ventricular hemorrhage (IVH) and necrotizing enterocolitis (NEC).[19]

Neonatal metabolic screening

By the mid-20th century, recognition of the utility of systematic prevention, confirmed by polio vaccines and the identification and treatment of congenital syphilis, leaded to universal Neonatal Screening programs.[20] The first test was called the Guthrie test and was introduced by Robert Guthrie, who developed a bacterial inhibition assay for phenylalanine together with the filter paper blood specimen system that made systematic screening possible.[21] Over the next decade, several other inborn errors of metabolism were added to the screening, such as maple-syrup urine disease, homocystinuria, galactosemia and congenital hypothyroidism, introduced in the early 1970s. Recently, with the development of electrospray tandem mass spectrometry, a single test is used to screen for a wide range of very rare disorders that have not been screened for previously.[22]

Jaundice: Over the past 40 y, there has been an enormous change in the cause of severe hyperbilirubinemia. Rhesus incompatibility was a major problem 40 years ago, resulting in the need to perform large num-

bers of exchange transfusions to prevent the development of kernicterus, whereas now has been virtually eradicated.[23] The mainstay of treatment is phototherapy. Since early discharge of term infants has become the norm, detecting and preventing severe hyperbilirubinemia makes the task of more difficult. The use of transcutaneous bilirubinometry to facilitate detection and lessens the need for blood tests. Norms for age-specific levels of total serum bilirubin, hour by hour from 18 to 168 h after birth have been published.[24]

All these discoveries and advances in neonatology have resulted in the reduction of infant mortality worldwide. A major contributor to the decline in infant mortality is the decline in neonatal deaths associated with low birthweights; in fact, neonatal survival or mortality is strongly related to the number of premature or LBW infants born. In the United States, the incidence of prematurity has increased in recent years: this is largely due to an increase in the number of multiple births, often the result of advances in assisted reproduction techniques that now are commonplace. Prematurity is responsible for 75% of perinatal mortality and more than 50% of long-term morbidity.[25] However, an infant born in 1950 with a birthweight <1000 grams had only a 10-15% chance of survival, while an infant born in 2008 with a birthweight <1000 grams had a >60% chance of surviving the neonatal period. The gestational age at which 50% of neonates survived decreased from 29 weeks (wks) in 1960 to 24 wks by the early 1990s. Unfortunately, the number of infants with disabilities has stayed approximately stable over time because of increased survival at lower gestational ages.[26] The majority of infants born very preterm now survive, but short-term consequences (RDS, IVH, periventricular leukomalacia-PVL, early- and late-onset sepsis, NEC) and long-term consequences (respiratory morbidity linked to bronchopulmonary dysplasia-BPD, visual impairments related to retinopathy of prematurity-ROP, neurodevelopmental and behavioral problems) remain a concern. With prematurity, the other leading causes of neonatal mortality and neurological disability are cerebral stroke and hypoxic-ischemic encephalopathy (HIE), more frequent in full-term babies. Therefore, today and in the future, further efforts must be made to improve neonatal and long-term outcomes.[27]

WHAT ABOUT TODAY?

To date, scientific evidences have shown that the environmental conditions experienced in early life can profoundly influence human biology and long-term health. This concept underlies the emerging interest toward the *developmental care* for preterm newborns, a range of strategies designed to reduce the stresses of the Neonatal Intensive Care Units (NICU). An unfavorable environment in the NICU can add to the prematurity-related problems and negatively affect the infant's growth, with the brain being particularly vulnerable; the preterm brain is in fact susceptible to a stressful environment and the detrimental effects of this stress could have short and long-term implications for compromised neurobehavioral development. Environmental modifications like reducing noise and light, minimal handling and giving longer rest periods, together with adequate pain control, could minimize the iatrogenic effects on the future health of the infant.[28]

However, another aspect of the early environmental impact on the future health of the newborn has been extensively studied in the last decades. More and more studies have confirmed the Barker's hypothesis or thrifty phenotype hypothesis, according to which environmental factors as poor nutrition in early life produces permanent changes in glucose-insulin metabolism and so predisposes to chronic adult diseases like metabolic syndrome and cardiovascular disease.[29] The concept of *fetal programming* has long been known: fetal programming occurs when the normal fetal development is disrupted by an abnormal insult applied to a critical point in intrauterine life; placenta assumes a crucial role in programming the intrauterine experience due to the adaptive changes in structure and function and so a crucial role in developmental plasticity.[30] Many complications of gestation such as preeclampsia, gestational diabetes and hypoxia can affect fetal programming, and recently an emerging area of research is the diet-induced inflammation in gestational tissues on fetal growth and development. Accumulating evidences now suggest that low-grade intrauterine inflammation might impair linear growth and adversely affect myogenesis and adipogenesis that might have lasting effects on offspring.[31] Epigenetic modulation represents the molecular mechanism that underlies the development of adverse health conditions in adulthood. In the past, it was thought that at the base of the development of many pathological conditions there were predominantly non-modifiable genetic factors, now we are instead aware of the importance of environmental factors (diet, toxic, drugs, microbiome, social and financial status and so on) that act through epigenetic modifications. These changes, especially occurring in the early period of life that is the one of major plasticity, contribute to the individual susceptibility to disease and can lead to transgenerational detrimental effects. Maternal nutritional constraint during pregnancy can alter the metabolic phenotype of the offspring by means of epigenetic regulation of specific genes, and this can be passed to the next generations,[32] but many other factors – maternal, fetal and placental – can influence the future vulnerability of the offspring. In a recent review, Feinberg argued that epigenetic changes are involved in normal development and human disease and proposed the term "epigenetic disease" to describe defects in the epigenome that are known to lead to disease. These de-

fects include changes in the localized or global density of DNA methylation, incorrect histone modifications or altered distribution or function of chromatin-modifying proteins that, in turn, lead to aberrant gene expression. According to Feinberg, defects in phenotypic plasticity or the cell's ability to change its behavior in response to internal or external environmental cues are the underlying theme of epigenetic disease; this can also be applied to common diseases with late-onset phenotypes that involve interactions between the epigenome, the genome, and the environment.[33]

According to recent research, the lowest common denominator seems to be the *oxidative stress* (*OS*). Chemical contaminants in water and diet, tobacco smoke, air pollution, gestational diabetes, hypertension and preeclampsia are all conditions that can produce an imbalance in the pro-oxidant/anti-oxidant system, leading to the increased production of free radicals (FRs) during gestation and so to fetal oxidative stress. Fetuses and newborns – especially preterm – are particularly susceptible to such an imbalance because of their exposure to conditions that can lead to a burden of FRs (ischemia, hypoxia-reperfusion, infections, inflammation, but also transfusions, drugs, hyperoxia) in association with insufficient scavenger systems.[32] FRs are highly reactive substances capable to start self-amplified chain reactions causing cellular dysfunction and damage to all components of the cell, including protein, lipids and DNA.[34] Biomarkers *in vivo* of oxidative damage are non protein-bound iron (NPBI, marker of potential OS), Advance Oxidation Protein Product (AOPP, marker of protein oxidation), isoprostanes (IsoPS, markers of lipid peroxidation deriving from arachidonic acid) and Isofurans (IsoFs, metabolically stable compounds forming at higher oxygen tension), Neuroprostanes (NPs, markers of neuronal oxidative damage deriving from lipid oxidation of docosahexaenoic acid-DHA) and Neurofurans (NFs).[34,35]

Many known or suspected causes of or conditions associated with impaired fetal growth or preterm birth have been associated with OS, so it may be the common link underlying the associations between adverse fetal growth or preterm birth and elevated risks of certain chronic diseases. The mechanisms of "oxidative stress programming" may be direct, through modulating gene expression, or indirect, through the effects of the oxidized molecules.[36] Some conditions of pregnancy are specific trigger for the overload of FRs: preeclampsia, intrauterine growth restriction, diabetes and maternal obesity. In fact F2-Isoprostanes (F2-IsoP), the main marker of arachidonic acid peroxidation, are higher in pregnancies with fetal growth restriction compared with pregnancies without, when dosed in amniotic fluid; they have a moderate power to distinguish fetal-growth-restricted pregnancies and so between adeguate- and small-for-gestational-age newborns.[37] Furthermore, also preterm premature rupture of membranes (pPROM) has been associated with OS, demonstrated by higher levels of F2-IsoP in amniotic fluid of mothers with pPROM compared with control pregnancies.[38] In the preterm neonate, tissue and organ damage involving kidneys, retina, lung, brain, and bowel has been related with elevated level of OS biomarkers in cord blood. In a recent study, the development of pathologies of prematurity like ROP, BPD, NEC and IVH was significantly associated with high cord blood levels of Total Hydroperoxide-TH, AOPP, and NBPI, thus leading to the hypothesis of "Free Radical-related Disease" (FRD) of prematurity.[38] In particular, plasma NBPI has demonstrated to be the best early predictive marker of neonatal brain damage, with a 100% sensitivity and 100% specificity for good neurodevelopmental outcome at 0-1.16 micro mol/L, and for poor neurodevelopmental outcome at values >15.2 micro mol/L.[38]

Not only preterm, but also full-term babies are particularly vulnerable to OS-related damage: OS seems to represent the underlying pathological mechanism even in neonatal asphyxia. In a recent study, newborns with severe asphyxia showed higher OS than those with mild asphyxia at birth, and AOPP was significantly associated with the severity of brain injury assessed by neuroimaging techniques (MRI), especially in males. The presence of an association between biomarkers of OS 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.[38]

All these evidences open the door to the ever-increasing research on antioxidant strategies. There are many antioxidants studied, especially for neonatal brain injury: iron chelators, FRs scavengers, inhibitors of lipid peroxidation, FRs reducing agents and many others. Among these, melatonin and docosahexaenoic acid are particularly manageable and promising according to studies on animal models, although further confirmations on humans are necessary.[39-42]

WHAT DOES THE FUTURE HOLD?

Many initiatives are already part of the present, but represent the future since will allow the increasing improvement of the quality of life when they arrive at the patient's bedside.

Some example are *gene therapy* that will consent to replace a defective gene that underlies a specific condition, or *biologic reporters*, light-emitting enzymes (luciferases) that can label genes and cells and could be used to monitor gene expression and immune therapies.[43]

The "*omics*" sciences represent perhaps the major area of growing interest and research in neonatology and paediatrics. Genomics, transcriptomics, proteomics and metabolomics represent all the complexity of biological systems and are going to replace traditional laboratory

methodologies thanks to their capacity to distinguish a single subject in normal conditions and in case of disease with a simultaneous non-invasive analysis of a large amount of data.[39] Metabolomics summarizes the gene-environment interactions, and consists of the quantitative analysis of a large number of low molecular mass metabolites involving substrates or products in metabolic pathways existing in all living systems. The analysis of the metabolic profile detectable in a human biological fluid (urine, plasma, also cord blood plasma, milk, stool…) allows to instantly identifying changes in the composition of endogenous and exogenous metabolites caused by the interaction between specific physiopathological states, gene expression, and environment.[44,45] From this point of view, metabolomics represent a sort of "identity card" of the individual in normal and pathological conditions, with each condition or disease presenting a specific discriminating set of metabolites.

In a recent study, distinct metabolic patterns were found between term infants and preterm infants, as well as between preterm infants of 23-32 week' gestation and those of 33-36 weeks' gestation. Individual metabolites discriminating between these groups were Hippurate, tryptophan, phenylalanine, malate, tyrosine, hydroxybutyrate, N-acetyl-glutamate and proline. Metabolomic analysis revealed distinct urinary profiles in newborns of different gestational ages and identified the discriminating metabolites.[46] In all sectors of the neonatology field there are ongoing studies aimed at validating the metabolic profiles correlated to specific neonatal pathological conditions, like neonatal asphyxia and hypoxic-ischemic encephalopathy, sepsis, necrotizing enterocolitis, acute kidney injury, bronchopulmonary dysplasia. For example, the urinary metabolic profile of newborns with HIE results significantly different from the one of healthy newborns.[46]

These recent advances lead to the concept of an *individualized and tailored medicine*. The application of omics methodologies in paediatrics and neonatology is crucial due to its unique ability to generate functional readouts of biological systems, but the clinical translation of this source of knowledge into clinical practices for neonatal health care requires proper addressing of the inherent inter-individual variability.[46] Therefore, further studies on this subject are certainly necessary but we can affirm with certainty that metabolomics is really the future, above all for our infants.

Remember: *"As neonatologists, we owe an enormous debt to our predecessors, but we are still faced with many challenges for the future"*[*A.G.S. Philip*].

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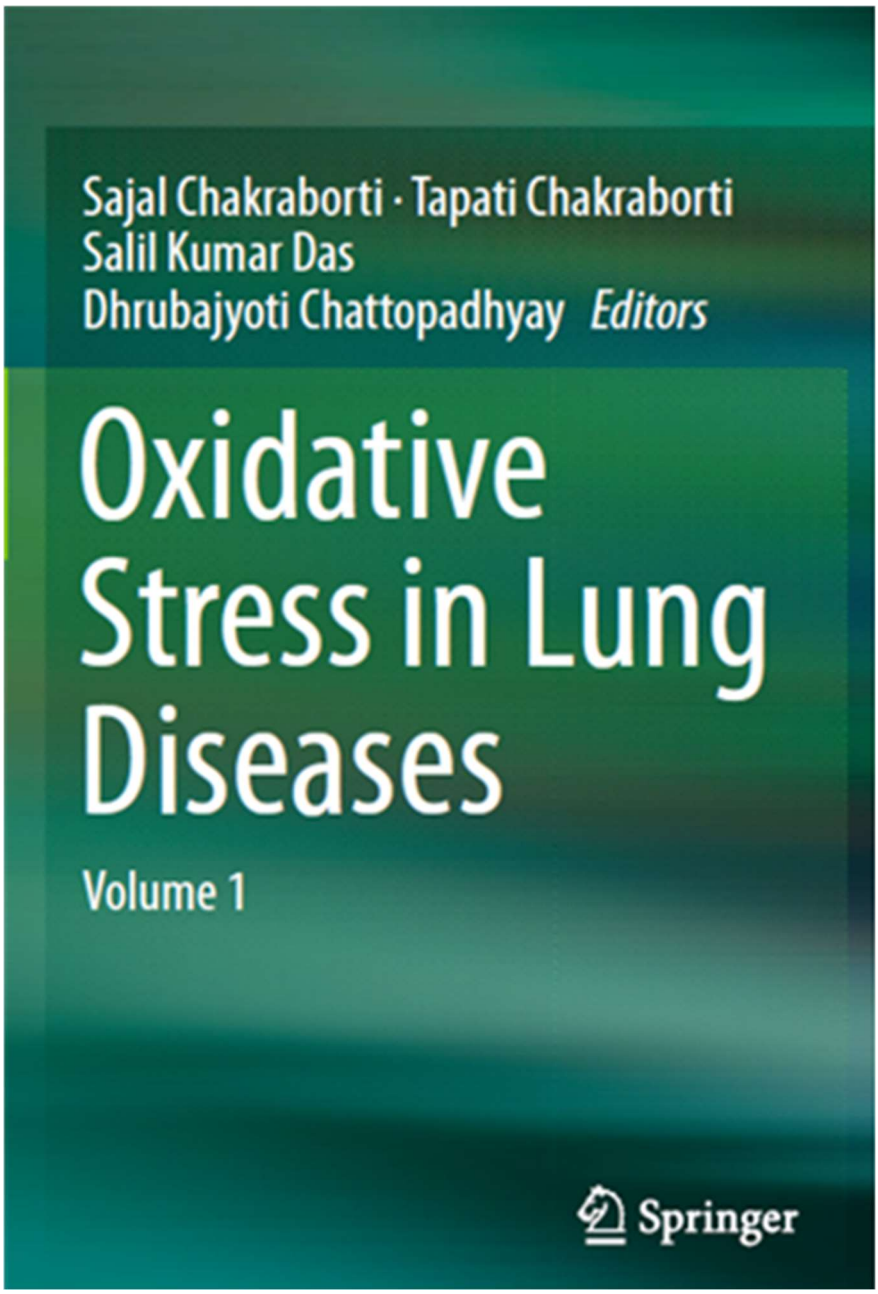
ANNEX 2:
Certificate of participation as speaker to the congress of the Clinical Biochemistry Study Group of Italian Society of Neonatology (SIN) "Novità dalla ricerca in biochimica clinica neonatale". Firenze, 16 Settembre 2020
Laschi Elisa. "*Il ruolo dei biomarcatori nella pratica clinica neonatale: dal laboratorio al letto del malato*".

ANNEX 3

Serafina Perrone, Elisa Laschi, Elisabetta Grande, Giuseppe Buonocore. *Bronchopulmonary dysplasia and oxidative stress in the newborn.* In: "Oxidative stress in lung disease (Vol-1)". Eds: Dr. Sajal Chakraborti, Dr. Tapati Chakraborti, Dr. Salil Kumar Das and Dr. Dhrubajyoti Chattopadhyay. Springer Nature Singapore Pte Ltd.2019. (https://doi.org/10.1007/978-981-13-8413-4_16. Published on 07/09/2019).



Bronchopulmonary Dysplasia and Oxidative Stress in the Newborn

16

Serafina Perrone, Elisa Laschi, Elisabetta Grande, and Giuseppe Buonocore

Abstract

Bronchopulmonary dysplasia (BPD) is a major cause of respiratory morbidity in preterm infants. The "new BPD" is the form currently observed in very preterm infants, related to disrupted and impaired lung development, and characterized by decreased alveolarization and impaired capillary development. Prematurity, oxygen toxicity, inflammation, mechanical ventilation, and surfactant deficiency are major determinants for BPD pathogenesis. Oxidative stress (OS) and inflammation are strictly interrelated originating a vicious circle that self-amplifies and ultimately leads to the damage of the immature lung. This chapter focuses on the role of OS on the lung injury and analyzes the most recent biomarkers in clinical studies. The comprehension of molecular basis of BPD is crucial for expanding the current possibilities for prevention and treatment and discovering new strategies for this condition.

Keywords

Bronchopulmonary dysplasia · Oxidative stress · Inflammation · Lung

16.1 Introduction

Bronchopulmonary dysplasia (BPD) is the major cause of chronic lung disease and pulmonary morbidity in preterm infants. In 1967, Northway first defined bronchopulmonary dysplasia as the presence of persistent respiratory signs and symptoms, the need for supplemental oxygen to treat hypoxemia associated with an abnormal chest radiograph at 36 weeks postmenstrual age (Northway et al. 1967; Kinsella et al. 2006).

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During the last decades, the advances in perinatal care, the prophylactic use of antenatal steroids, and surfactant replacement therapy for respiratory distress syndrome (RDS) improved the rate of mortality due to this condition but not the overall incidence of BPD and modified its pathophysiology and its clinical presentation.

The "old BPD" or "classic BPD" was considered a lung injury occurring in modestly preterm babies, born between 29 and 32 weeks of postmenstrual age, and related to surfactant deficiency and the need for oxygen supplementation and invasive ventilation; classic BPD was mainly characterized by inflammation, fibrosis, and scarring. In contrast, the "new BPD" currently occurs in younger preterm babies below 29 weeks PMA and can be considered a consequence of disrupted and impaired lung development; new BPD is mainly characterized by decreased alveolarization and impaired capillary development (Perrone et al. 2018; McEvoy and Aschner 2015).

Consequently, the definition of BPD has been very challenging over the years, and despite extensive studies, it remains extremely heterogeneous up to now. In 2000, a workshop organized by the National Institute of Child Health and Human Development (NICHD), the Nation Heart, Lung and Blood Institute (NHLBI), and the Office of Rare Disease (ORD) reviewed the definition of BPD and detailed diagnostic criteria for this condition based on gestational age and severity (Jobe and Bancalari 2001). In particular, it scored the severity of the disorder according to the gestational age less or more than 32 weeks PMA and to the level of respiratory support needed at 28–56 days of life and near term at 36 weeks PMA (Table 16.1).

During the last decade, despite the efforts for a consensus definition, published reports are still highly variable in terms of BPD definition with marked difference in identifying the risk of poor lung and neurodevelopmental outcome (Hines et al. 2017) and wide a variation in reporting incidence of BPD. In addition, the current definitions of the disease do not take into account the recent changes in respiratory management (such as the use of high flow nasal cannula), leading to an

Table 16.1 Definition of BPD, modified from (Jobe and Bancalari 2001)

Gestational age	< 32 weeks	≥ 32 weeks
Time point of assessment	36 weeks PMA or discharge to home (whichever comes first)	28 days but <56 days postnatal age or discharge to home (whichever comes first)
Definition	Treatment with oxygen >21% for at least 28 days +	
Mild BPD	Breathing room air at 36 weeks PMA or discharge (whichever comes first)	Breathing room air by 56 days postnatal age or discharge (whichever comes first)
Moderate BPD	Need for <30% oxygen at 36 weeks PMA or discharge (whichever comes first)	Need for <30% oxygen by 56 days postnatal age or discharge (whichever comes first)
Severe BPD	Need for ≥30% oxygen and/or positive pressure (PPV or NCPAP) at 36 weeks PMA or discharge (whichever comes first)	Need for ≥30% oxygen and/or positive pressure (PPV or NCPAP) by 56 days postnatal age or discharge (whichever comes first)

PMA: postmenstrual age

underestimation and a misclassification of the pathology (Kalikkot Thekkeveedu et al. 2017).

16.2 Epidemiology

It is difficult to establish accurately the incidence of BPD that change according to the clinical practice, to the definition used and to the ethnicity. Overall BPD affects almost 10,000–15,000 infants annually in the United States (Jensen and Wright 2018).

Furthermore, the risk of BPD is inversely related to gestational age and to the birth weight. According to the data from the Vermont Oxford Network, the rates of BPD vary from 12% to 32% among infants born less than 32 weeks gestation. Infants with birth weight less than 1000 g are the most susceptible to develop this condition: previous studies demonstrated that 75% of affected babies weighed less than 1000 gr at birth, but only 5% weighed over 1500 gr at birth (Jensen and Schmidt 2014).

16.3 Pathogenesis

The pathogenesis of BPD is complex and multifactorial. Lung injury in the neonatal period has multiple etiologic factors (genetic, metabolic, hemodynamic, nutritional, mechanical, and infectious) that act in a synergic way (Perrone et al. 2012). Prematurity, oxygen toxicity, inflammation, mechanical ventilation, and surfactant deficiency are all major determinants for the disease (Perrone et al. 2018). Recent hypothesis postulated a two-hit model that could lead to the appearance of BPD: the interaction between inadequate lung development due to several antenatal factors (genetic susceptibility, intrauterine growth restriction, maternal infections, or cigarette smoking) and postnatal damage (related to infections and sepsis, mechanical ventilation, and oxidative stress due to the exposure to a huge amount of free radicals, FRs). According to this model, the severity of the clinical picture depends on the efficacy of repair and antioxidant mechanisms: the airway remodeling is linked to a suboptimal repair and to the subsequent development of chronic lung disease (Kalikkot Thekkeveedu et al. 2017). Inflammation and oxidative stress are strictly interrelated in the pathogenesis of BPD, and they originate a vicious circle that self-amplifies and leads to the damage of the immature lung (Fig. 16.1).

16.3.1 Oxidative Stress in the Perinatal Period: A Role for BPD

Free radical reactions are a normal occurrence in living organisms, and free radicals (FRs) are deeply involved in signaling molecules to regulate a wide variety of physiological events (Perrone et al. 2018). The production of superoxide anion is the first stage of a physiological mechanism of host defense, followed by the production of

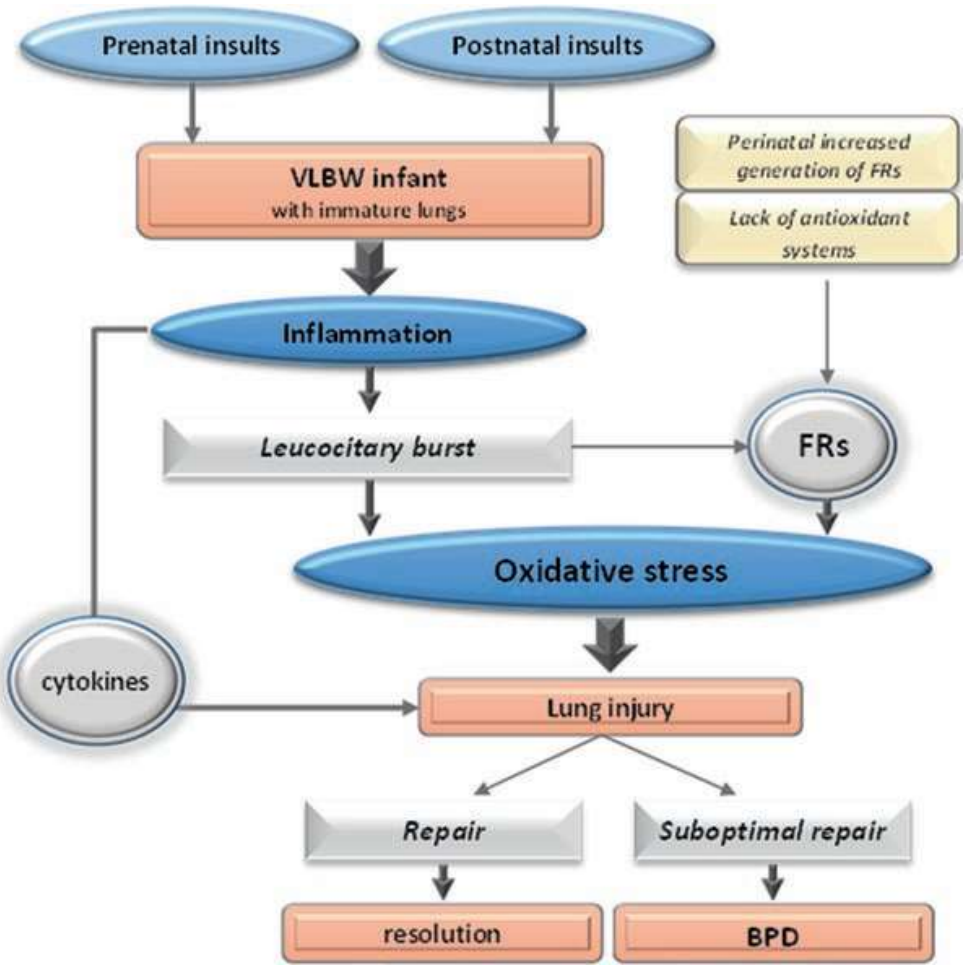

Fig. 16.1 Physiopathological mechanisms of BPD: role of inflammation and oxidative stress VLBW, very-low-birth-weight; FRs, free radicals

other reactive species, such as hydrogen peroxide (H_2O_2) by superoxide H_2O_2 dismutase (SOD), hydroxyl radicals (OH-) catalyzed by transition metals, and hypochlorous acid (HOCl-) by myeloperoxidase. These substances contribute normally to killing bacteria but, on the other side, favor tissue damage and increase capillary permeability, contributing to the passage of pro-inflammatory cytokines. Many of those increase the expression of inducible nitric oxide synthase (NOS), which forms nitric oxide (NO); NO combine with superoxide radicals to produce peroxynitrite that can form other potentially damaging metabolites such as hydroxyl radicals, nitrogen dioxide (NO_2), and nitrogen dioxide radical (NO_2+) (Perrone et al. 2012). Other sources of increased FRs generation are hyperoxia, hypoxia, ischemia, blood transfusion increased levels of nonprotein-bound iron, xenobiotics, and drugs. The antioxidant enzymes like SOD, catalase, and glutathione synthetase (GSH) have usually the capacity to scavenge the levels of FRs produced in physiological conditions, but under ischemic conditions and especially in preterm infants they fail to protect tissues from oxidative damage because of the overproduction of oxygen radicals and consumptions of antioxidant defenses (Perrone et al. 2018). The imbalance between oxidant and antioxidants with predominance of one or the other has

been called oxidative stress (OS). Over the last decades, emerging data have suggested that OS is involved in the development of BPD and that the lung injury process leading to BPD occurs within hours to days from delivery with the oxidation representing a major contributor to the process (Perrone et al. 2012).

Since 1996, several studies on murine model detected an inhibition of fetal lung growth after exposure to high oxygen concentrations, partially reversible using low-molecular-weight SOD mimetic; these results suggested that superoxide anion and possibly hydroxyl radical are the oxygen-centered FRs most likely responsible for the growth effects of hyperoxia on mouse fetal lung morphogenesis (Wilborn et al. 1996). More recently, Datta et al. showed that early (day 0) but not late (day 4) postnatal hyperoxia compromises the lung development decreasing the alveolar count and septal count, increasing distal artery muscularization, and inducing right ventricular hypertrophy. All these alterations were attenuated by the use of antioxidants during the early hyperoxia. In addition, the study group demonstrated that hyperoxia-induced NOX1 enzyme could amplify the FRs signaling contributing to the observed defects in lung and pulmonary vascular development (Datta et al. 2015). NOX1-deficient mice presented hyperoxia-induced lung injury due to cell death of the alveolo-capillary barrier via c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) pathways, two mitogen-activated protein kinases involved in cell death signaling (Carnesecchi et al. 2009).

Hyperoxia also induces the fibrotic process in newborn with BPD (Perrone et al. 2012). Hyperoxia-induced FRs could interfere with surfactant production inducing an intracellular accumulation of surfactant proteins SP-A, SP-B, and SP-C_{85} (SPC) (Perrone et al. 2012; Zhang et al. 2015) and reducing surfactant phospholipid production (Perrone et al. 2012). FRs could also inhibit the trans-differentiation of alveolar epithelial cells II (AEC II) into alveolar epithelial cells I (AEC I) (Zhang et al. 2016). AEC II are the most important stem cells in lung tissue and play a crucial role in the regulation of lung tissue growth, maturation, wound-repair process, and lung fluid homeostasis; proliferation and differentiation of these cells are the determinant to regulate the post-injury repair of alveolar epithelial structures and functions. AEC II are physiologically converted to AEC I that secrete bioactive substances to restore alveolo-capillary barrier integrity and maintain alveolar function: hyperoxia-induced OS determines excessive apoptosis of AECII and inhibits their proliferation (Wang and Dong 2018).

Pulmonary remodeling but also vascular remodeling induced by hyperoxia represents the histopathological basis of new BPD (Carnesecchi et al. 2009). Delaney et al. postulated that OS and the consumption of antioxidant enzymes as SOD could be responsible for the appearance of pulmonary hypertension disrupting the VEGF/VEGFR2/nitric oxide (NO) pathway.

VEGF (*vascular endothelial growth factor)* signaling is crucial for normal alveolar and vascular growth; previous studies have shown that VEGF and VEGFR2 (*vascular endothelial growth factor receptor type 2)* are decreased in infants who died with BPD, and in models of BPD and pulmonary hypertension, disruption of VEGF signaling contributes to pathogenesis. In the longer period, FRs could reduce

the levels of VEGFR2 indispensable for angiogenesis in the fetal and neonatal lung by phosphorylation (Delaney et al. 2015).

Other studies have found that increased FRs production in the pulmonary vasculature influences redox-sensitive signaling pathways in pulmonary vascular cells, leading to activation of redox-sensitive transcription factors and increasing the expression of many proteins involved in pulmonary vascular remodeling, inflammation, and altered bronchial reactivity (serine/threonine kinases MAPK, ROCK, Akt) (Freund-Michel et al. 2013).

Emerging theories indicate also that endoplasmic reticulum stress could be crucial for the cell survival. At the same time, FRs cause protein misfolding and activate the transcription of factors as GRP78 and CHOP that are involved in the apoptosis pathway (Lu et al. 2015).

16.3.2 Oxidative Stress and Inflammation

An important common denominator in the pathogenesis of BPD is inflammation. Both antenatal and postnatal causative factors activate several inflammatory cascades, determining on an immature immune system an impairment in cytokines' production and lung uptake of inflammatory cells. Cytokines mediate acute lung injury, exacerbate ventilator-associated lung injury, modulate host defense, and contribute to normal lung development. In a study conducted on over 1000 extremely low-birth-weight infants, those who developed BPD or died had elevated IL-8 accompanied by a relative predominance of T-helper2 cytokines (IL-10, IL-6) in comparison to T-helper1 cytokines (TNF-β) or T-cell products (RANTES), although some Th1 cytokines (IL-1 β, IFN-γ) were also elevated and associated with BPD or death. This pattern of cytokines reveals an increased early neutrophil influx and a relative decrease in effector T-cells and confirms the hypothesis that BPD may be associated with an impairment in the transition from the innate immune response mediated by neutrophils to the adaptive immune response mediated by T-lymphocytes (Ambalavanan et al. 2009). Higher levels of IL-1β, IL-8, and IL-6 in tracheal aspirates at birth are predictive for later BPD, suggesting that early exposure of the premature lung to inflammation is important to the development of BPD and that pro-inflammatory cytokines appear to mediate this process (Ryan et al. 2008). In an early stage of development, cytokines stimulate the infiltration of lung interstitial space by polymorphonucleated cells (PMN) and macrophages (MAC) and then lead to the overproduction of metalloproteinases (MMPs) that play a key role in disruptive processes with simplification of alveolar tree and activation of fibrotic processes. Furthermore, the persistence of lung inflammation and release of inflammatory mediators contribute to the overexpression of adhesion molecules by endothelial cells resulting in trans-endothelial cytokine migration (Perrone et al. 2018) and by smooth muscle cells with consequent appearance of pulmonary hypertension and bronchial over-reactivity (Ryan et al. 2008). The entire molecular pathway is not fully understood, but recent advances suggest that oxidative stress is the

final common endpoint for a complex convergence of events mediated by FRs generation (Wang and Dong 2018).

This process involves the activation of inflammatory cells ("leukocitary burst") triggered by antenatal or postnatal infections or by aspecific stimuli like lung stretching during mechanical ventilation, with subsequent release of a large amount of oxygen radicals and proteases (Perrone et al. 2012; 2018). Indeed, the inflammatory cascade is associated with the production of FRs, with neutrophil generating superoxide anions, hydrogen peroxide, and hydroxyl anions. The physiologic deficiency of antioxidant systems contributes to oxidative stress (Wang and Dong 2018). FRs can directly damage all biological molecules but especially proteins, lipids, and nucleic acid and cause cell death through a variety of patterns. They increase the amount of ceramides to induce apoptosis by lipid peroxidation, affect the activity of various enzymes by processes of protein oxidation and nytrosilation, can modify the DNA structure, and activate multiple signal transduction pathways in cells, promoting the transmission of cytokines, growth factors, and calcium signals. All of these mechanisms contribute to maintain the previously activated damaging processes (Saugstad 2003).

16.4 Biomarkers of Oxidative Stress in Clinical Study

Despite recent advances on the role of FRs in murine models of BPD, our knowledge about their action on fetal and newborn lungs is less strong. Newborns and especially preterm infants are particularly vulnerable to the oxidative damage because of their major production of FRs and their weak antioxidant systems with lower possibility to scavenge FRs in excess.

Physiologically, a complex relationship between oxidant and antioxidant systems exists, and the imbalance between these two components with predominance of the first one could activate several processes – epigenetic and enzymatic – that mediate a remodeling of the small airways and the lung vascular bed of the newborn.

However, a useful recognized biomarker (on bronchoalveolar lavage (BAL), plasma, or urine) that can correlate with the risk of developing BPD or that can predict the severity of lung injury and the long-term respiratory impairment is not yet know (Perrone et al. 2018). During the last years, several metabolites have been studied as indicators of OS in respiratory distress syndrome and chronic lung disease.

In 2015, Negi et al. detected higher levels of plasma *malondialdehyde (MDA)*, *protein carbonyl,* and *8-hydroxy-2-deoxyguanosine (8-OHdG)* in the umbilical cord blood of a population of low-birth-weight preterm babies who developed respiratory distress syndrome compared to those who did not develop the disease, with a significant difference between the groups. MDA is a marker of lipid peroxidation as final product of polyunsaturated fatty acids peroxidation; protein carbonyls are markers of early protein oxidation; 8-OHdG can be considered a marker of oxidative DNA damage. (Negi et al. 2014). FRs could lead to an increased permeability

of vascular bed, with shift of proteins in the interstitial spaces and consequent storage of oxidized proteins and fat; at the same time the cellular damage may promote DNA modification and activation of cell death pathways. How the acute lung injury could be related to the chronic modifications that underpin the structural and functional changes in BPD is up to date ongoing on study.

Studies on short-term measurements demonstrated increased levels of OS products in infants with BPD. Joung et al. measured the urinary levels of *8-hydroxydeoxyguanosine (8-OHdG)* and *leukotriene E4* (*LTE4*, product of degradation of the phospholipid bilayer of cell membrane), oxidative and inflammatory stress markers, respectively, in a group of very-low-birth-weight (≤1250 g) infants ≤30 weeks GA. The authors reported that the urinary excretion of 8-OHdG and LTE4 in newborns with BPD was higher respect to the ones without disease. The first biomarker was associated to the clinical phenotype of the old BPD, while the second one was associated with the phenotype of the new or atypical BPD, characterized by a more marked bronchial reactivity. Collaterally, they showed that the level of urinary LTE4 on day 1 was greater than the normal adult value in both the no/mild BPD and moderate/severe BPD groups, supporting the hypothesis that prenatal inflammatory insults may play a role in the pathogenesis of the disease. Furthermore, the persistence of high excretion of 8-OHdG on the 3th day of life was related to the duration of mechanical ventilation (Joung et al. 2011)**.** Conversely, Moore et al. in 2016 demonstrated that the infants with lower urinary 8-OHdG levels on day 7 of life required higher levels of supplemental oxygen during all the study period. This difference could be partially attributable to the lower gestational age of newborns that developed BPD, but the authors supposed that it could be explained by the immaturity of the enzymes that recognize and remove the oxidized lesions (Moore et al. 2016)**.** The results of this study documented the extreme complexity of the role of oxidative stress in the pathogenesis of disease.

A recent study measured and compared *glutathione (GSH)* and *lipid peroxidation products (LOOH)* concentrations in the bronchoalveolar lavage fluid (BALF) of a group of very-low-birth-weight newborns with and without BPD. The first group showed significantly lower antioxidant defense (GSH levels) and higher levels of LOOH than the second one. Furthermore, LOOH were associated with respiratory support parameters such as the length of mechanical ventilation, supporting the role of volu/barotrauma in the activation of inflammatory cascade with consequent amplification of lung damage (Fabiano et al. 2016)**.**

In 2015, Kuligowski et al. measured the levels of lipid peroxidation metabolites isoprostanes (IsoPs), isofurans (IsoFs), neuroprostanes (NeuroPs), and neurofurans (NeuroFs) on serial urinary samples in a population of preterm infants ≤32 weeks gestational age and compared the levels between babies who later developed BPD and controls. IsoPs and IsoFs are reliable and stable compounds originating from oxidation of arachidonic acid, in condition of normoxia or hyperoxia, respectively; in a similar manner, NeuroPs and NeuroFs originate from lipid peroxidation of docosahexaenoic acid (DHA) in brain tissue. The authors detected that increased urinary elimination of isofurans in the early perinatal period (in the first 4 days after birth) was related to the development of BPD **(Kuligowski et al.** 2015**).**

Matthews et al. have shown that increased plasma concentrations of isoprostanes (F2-IsoPs) in the first month of life are associated with an increase in the need of respiratory support at term equivalent age of preterm infants. The authors also reported a worse neurodevelopmental outcome at 12 months corrected age in extremely preterm infants with high isoprostanes levels (Matthews et al. 2016).

Various pro-inflammatory cytokines like IL-1β, IL-6, IL-8, IL-10, IL-18, TNF-α, and IFN-γ have been shown to correlate with an increased risk of BPD in preterm babies (Ambalavanan et al. 2009; Ryan et al. 2008; D'Angio et al. 2016; Jónsson et al. 1997). A longitudinal analysis studied the main pro-inflammatory cytokines (IL-6, IL-8, and *granulocyte-colony stimulating factor*, G-CSF) in different times point between birth and 42 days of age. In infants with BPD, inflammation occurred shortly after birth with gradual but not complete attenuation in the first neonatal period, and corticosteroids seemed to be able to modulate the process (Leroy et al. 2018).

The link between interleukin pathway and OS biomarkers has been recently reported. IL-6 and 8-OHdG from serum and tracheal aspirate were higher in very-low-birth-weight (VLBW) infants with BPD than in babies without BPD on the first day after birth. The IL-6 and 8-OHdG levels in tracheal aspirate fluid were also persistently increased on postnatal day 28th in the BPD group, confirming the hypothesis that the persistence of the inflammation can be an important mechanism in the pathogenesis of BPD (Hsiao et al. 2017).

16.5 Antioxidant Drugs and BPD Prevention

Another important pathogenic factor of BPD is the peculiar deficiency of antioxidant systems in preterm newborns (Perrone et al. 2010). Giusti et al. identified in the association between single-nucleotide polymorphisms (SNP) of genes encoding for superoxide dismutase SOD1, SOD2, SOD3, and CAT a protective or risk factor for developing prematurity complications, including BPD (Giusti et al. 2012). Moreover, an increased rate of BPD was demonstrated in infants with SNP of NAD(P)H: quinone oxidoreductase 1 (NQO1), a flavoprotein enzyme that catalyzes two electrons reduction of a variety of substrates including quinones (Sampath et al. 2015).

The beneficial effects of antioxidant therapies in the prevention of FRs-mediated lung injury are still controversial, because the consequences of prematurity are multifactorial and many prenatal and perinatal insults could influence their clinical course. Therefore, data from clinical trials are up to date insufficient.

The main data available in literature concern melatonin (MT), antioxidant enzymes supplementation (AOEs), and vitamins.

Melatonin is the main product of the pineal gland and has antioxidant, antiapoptotic, and anti-inflammatory properties; it inhibits the NOS and lipid peroxidation and favors the transcription of antioxidant enzymes. In particular, MT exerts a double antioxidant activity: it scavenges directly ROS and induces the expression on glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase

(Poggi and Dani 2014). Previous studies demonstrated its role in preventing brain injury during hypoxic-ischemic encephalopathy and the possibility to influence positively the clinical course in septic newborns reducing the concentrations of lipid peroxidation products (Gitto et al. 2001), but there are fewer studies considering its efficacy in preventing chronic lung disease of prematurity. Melatonin seems to be able to reduce IL-6, IL-8, and TNF-α concentrations and nitrite/nitrate levels in serum in the first hours of life and in the early postnatal period of neonates with respiratory distress (Gitto et al. 2004). Newborns mechanically ventilated with pressure support ventilation mode with guarantee volume and receiving melatonin presented a greater reduction of serum levels of inflammatory cytokines than did newborns ventilated in other modes or not receiving melatonin. Furthermore, MT seems to reduce the accumulation of leukocytes into the lung manifested by the reduction in myeloperoxidase activity, and this other effect contributes to limit the oxidative damage predisposing to BPD (Gitto et al. 2013). In recent animal studies, melatonin seems to have positive effects even upon the vascular side: postnatal administration of MT blunts the cardiopulmonary response to hypoxia, reduces the pathological vascular remodeling, and increases angiogenesis in pulmonary hypertensive neonatal lambs, improving the pulmonary vascular structure and function in the neonatal period under chronic hypoxia (Astorga et al. 2018).

In animal models the lack of SOD leads to impaired alveolar development, pulmonary hypertension, and disrupted pulmonary vascularization (Delaney et al. 2015), while transgenic expression of SOD preserves the AEC II proliferation and attenuate hyperoxic induction of vascular remodeling (Auten et al. 2006; Nozik-Grayck et al. 2008). Moreover, exogenous supplementation of SOD could increase in lung tissue the bioavailability of NO and downregulate the NF-KB pathway, exerting a protective role on lung development. Actually, there is not a clear evidence of efficacy of SOD supplementation for the newborns. Endotracheal administration of recombinant human SOD (rhSOD) has been proved to reduce lung injury in preterm newborns receiving mechanical ventilation for RDS (Poggi and Dani 2014): its recombinant form has a short half-life; consequently it seems to be able to reduce lung inflammation and the oxygen requirement in respiratory distress syndrome if administered in single doses. Therefore, repeated doses do not seem to modify the oxygen dependency and the duration of mechanical ventilation but to exert a positive influence on the frequency of respiratory problems after the discharge (Suresh et al. 2001).

A possible application vitamin A in preventing BPD was argued observing that vitamin A deficiency in animal model linked to changes in the respiratory tract epithelium including necrotizing tracheobronchiolitis and squamous metaplasia; all these modifications were reversible by restoring the vitamin storage (Wardle et al. 2001).

In animal models exposed to hyperoxia, vitamin A and its metabolites lowered lung injury (considered as epithelial injury, hemorrhage, and macrophages infiltrate), and retinoic acid supplementation prevented the hyperoxia-induced increase of IL-1β, IL-6, and TNF-α (James et al. 2010). In 2016, a Cochrane review of the studies in preterm infants unlighted the efficacy of this vitamin in reducing the need

of oxygen therapy at terms and 28 days after the discharge, without significantly modifying the neurodevelopmental outcome of the examined population at 18 and 22 months of corrected age (Darlow et al. 2016).

16.6 BPD Treatment

Considering its multifactorial pathogenesis, BPD requires a multidisciplinary approach for an effective treatment.

Despite the several therapeutic strategies proposed over the time (bronchodilators, diuretics, pulmonary vasodilators), the use of systemic corticosteroids is the only one related to a significant reduction of the incidence of BPD. However, this treatment prolonged on the time is associated with an impairment of brain maturation and worse developmental outcome (Iyengar and Davis 2015). In a meta analysis, Shah et collegues demonstrated that early and late administration of systemic corticosteroids reduce the risk of BPD, facilitate extubation, and reduce the incidence of patent ductus arteriosus but at the same time increase the risk of gastrointestinal bleeding, growth failure, and neurological impairment in preschooler age. (Shah et al. 2017a).

More debated is the usefulness of airway-administered corticosteroids, in particular budesonide.

According to the last Cochrane review about the topic, the use of nebulized budesonide does not modify significantly the risk of chronic lung disease and the oxygen dependency (Shah et al. 2017b).

The Neonatal European Study of Inhaled Steroids (NEUROSIS) Trial Group have recently reported a significant reduction of incidence of BPD in infants treated by inhaled budesonide respect the ones who received placebo (Bassler et al. 2015). The subsequent analysis on long-term follow-up did not show a significantly difference in neurodevelopmental disability between the groups of study, but the mortality rate was higher among preterm infants who received budesonide (Bassler et al. 2018).

The mechanism by which the steroid therapy reduces lung inflammation modulating the immune response has been extensively studied, while the relationship between the use of corticosteroids and the oxidative damage is less known. In a cohort of extremely premature newborns, Vento et al. detected an association between the antenatal steroids supplementation and reduction of postnatal OS conditions (Vento et al. 2009). In a more recent study, Sandal et al. detected a significantly reduction in total oxidant status in infants with BPD after the treatment with hydrocortisone given orally. The authors measured the total oxidant status (TOS) and total antioxidant capacity (TAC) levels of a group of preterm infants before and 1 week after the hydrocortisone therapy and calculated oxidative stress index (OSI) levels. Following the treatment with hydrocortisone, they observed a statistically significant decrease in TOS and OSI index and an increase in TAC levels in comparison with pre-treatment levels (Sandal et al. 2013). This is the only study in literature considering specifically the effects of steroid on oxidative stress, and then

we can only hypothesize that steroids could block a cascade of events (which include cellular recruitment, cytokines production, and enzymatic activation) having as common final endpoint the FRs, the true effectors of tissue damage.

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VALUTAZIONE DELLA DENSITÀ MINERALE OSSEA IN UN GRUPPO DI GIOVANI ADULTI NATI PRETERMINE: IL FOLLOW-UP MULTISCIPLINARE.

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Background e obiettivi: La prematurità costituisce un fattore di rischio associato a una riduzione della densità minerale (DM) ossea nell'età adulta. Tale condizione è connessa ad un aumentato rischio di fratture invalidanti, che influiscono inevitabilmente sulla vita del soggetto, rappresentando un significativo problema medico e sociale. Lo scopo di questo studio è valutare l'impatto della prematurità sulla densità minerale ossea in età giovane adulta e indagare gli eventuali fattori di rischio associati.
Materiali e Metodi: Sono stati arruolati 74 pazienti di età compresa tra 16 e 23 anni: di questi, 55 (età media: 20,2±3,4 anni) erano nati pretermine con età gestazionale media di 30,5±2,4 settimane e peso alla nascita 1398±301,3 g, mentre 19 (età media: 20,4±1,5 anni) nati a termine di gestazione. Di tutti i pazienti sono state raccolte informazioni relative al periodo neonatale (dati auxologici, durata della nutrizione parenterale, durata della degenza in ospedale e durata della ventilazione meccanica) e all'età adulta (altezza, body mass index, partecipazione ad attività sportive). Sono stati inoltre valutati i livelli di colesterolo totale, colesterolo HDL e LDL in tutta la popolazione in studio. La DM ossea è stata valutata utilizzando l'ultrasonografia quantitativa (DBM Sonic BP, Igea) misurando: Ad-SoS in m/s (Amplitude Dependent-Speed of Sound, la velocità della propagazione del segnale ultrasonoro attraverso la falange), BTT in µsec (Bone Trasmission Time, il tempo impiegato dall'ultrasuono per attraversare l'osso), ed i corrispondenti z-scores.
Risultati: Nei giovani adulti Ad-SoS correla con l'età attuale (p=0,003, r=0,345) e con l'altezza attuale (p=0,030, r=0,253); BTT correla con l'età attuale (p=0,001, r=0,386) e col peso attuale (p=0,001, r=0,379). Nel nostro gruppo in studio, non sono state evidenziate differenze significative tra i giovani adulti nati a termine e pretermine riguardo ai valori assoluti di Ad-SoS e BTT e z-scores. È stata riscontrata invece un'associazione statisticamente significativa tra BTT-z score, AD-SoS z-score e valori di colesterolo totale e colesterolo LDL (p=0.002). Nel gruppo dei giovani adulti nati pretermine la regressione lineare univariata ha rilevato un'associazione tra BTT e durata della degenza in ospedale (p<0.0001), peso alla nascita (p<0.0001), e l'essere nato piccolo per età gestazionale(SGA) (p<0.0001). Il BTT score è significativamente più basso nei giovani adulti nati SGA rispetto ainati di peso adeguato per età gestazionale (-0.48 + 1.11 vs 0.22 + 1.07, p =0.03). La regressione multivariata ha mostrato una associazione statisticamente significativa tra BTT z-score attuale e l'esser nato SGA (p=0,033, CI 95% = -1.048; -0.068).
Conclusioni: In età giovane adulta la DM ossea è correlata con età e peso attuali. Maggiori sono i livelli di colesterolo totale e LDL, minore è la DM ossea. I giovani adulti nati pretermine piccoli per età gestazionale hanno una minore DM ossea rispetto ai coetanei nati a termine. Considerate le differenze riscontrate nei pretermine SGA, i dati suggeriscono che l'ambiente intrauterino nel quale il feto si sviluppa e la nutrizione abbiano un ruolo cruciale nella mineralizzazione ossea dell'età adulta
Ulteriori studi si rendono necessari per valutare quali siano i fattori che favoriscono il recupero della DM ossea nei nati pretermine e le tempistiche in cui tale recupero si verifica, al fine di ottimizzare gli interventi di prevenzione secondo un approccio sartoriale e quanto più precoce possibile.

ANNEX 5

Laschi E., Nanni G., Giordano M., Muraca M.C., Palombo D., Buonocore G., Perrone S. *The moderate and the late preterm infant: comparison on neonatal outcomes.* 3rd jENS - Congress of joint European Neonatal Societies, Maastricht 17-21 September 2019 (poster).

The moderate and the late preterm infant: comparison on neonatal outcomes

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Background

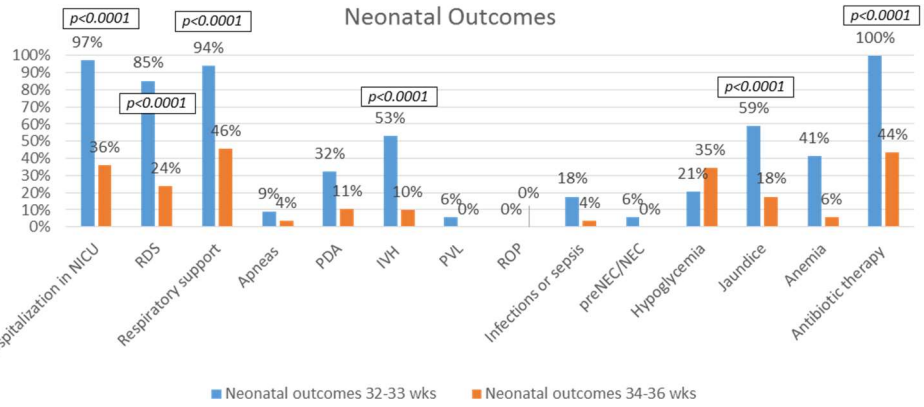
Moderate preterm (MPT, 32-33 weeks gestational age-GA) and late preterm infants (LPT, 34-36 wks GA) represent over 80% of preterm births, but are overall less known than those born at term and less studied than those born at lower gestational age. Since the maturational development takes place along the gestation continuum, each week of GA at birth can affect differences in the neonatal outcome of these infants and thus influence their management.

Materials and methods

An observational study on all live births of GA 32-36 weeks in a single III level center (Azienda Ospedaliera Universitaria Senese) in the years 2016-2017 was conducted. The aim of our study was to evaluate the short-term outcomes of MPT and LPT, with particular reference to the differences between the two groups related to care management and the frequency of the most common neonatal pathologies. The data concerning the obstetric history and the neonatal course were collected from the medical records of hospitalization; the auxological parameters at birth and discharge were calculated with reference to INeS neonatal anthropometric charts.

Results

Study population consisted of 176 infants (7.9% of all births; 34 MPT, 142 LPT). Significant differences emerged between the two groups regarding the following outcomes: need for resuscitation at birth (70.5% vs 29.5%); hospitalization in Neonatal Intensive Care Unit (NICU; 97% vs 35.9%); duration of admission to NICU (10.5 vs 1.5 days) and of overall hospitalization (28 vs 15 days); neonatal respiratory distress syndrome (85.2% vs 23.9%); need for any respiratory support (94.1% vs 45.7%); intraventricular hemorrhage of any degree (52.9% vs 9.8%); jaundice treated with phototherapy (55.8% vs 16.9%); iron supplementation (79.4% vs 7%); antibiotic therapy (100% vs 43.6%). Auxological parameters were significantly different between the groups, as well as the need for any nutritional support, the beginning of enteral feeding and the time to reach enteral and oral autonomy ($p<0.0001$).



Conclusions

Moderate preterm infants are at greater risk of unfavorable neonatal outcome compared to late preterm infants. In fact, the moderate preterms seem to behave more similarly to those born with a lower gestational age compared to the more "mature" LPT, although they also need particular attention and greater assistance, especially with regard to feeding methods.

ANNEX 6

Laschi E., Nanni G., Giordano M., Muraca M.C., Palombo D., Buonocore G., Perrone S. *The auxological outcome in the first year of life of the moderate and late preterm infants*. 3rd jENS- Congress of joint European Neonatal Societies, Maastricht 17-21 September 2019 (poster).

The auxological outcome in the first year of life of the moderate and late preterm infants.

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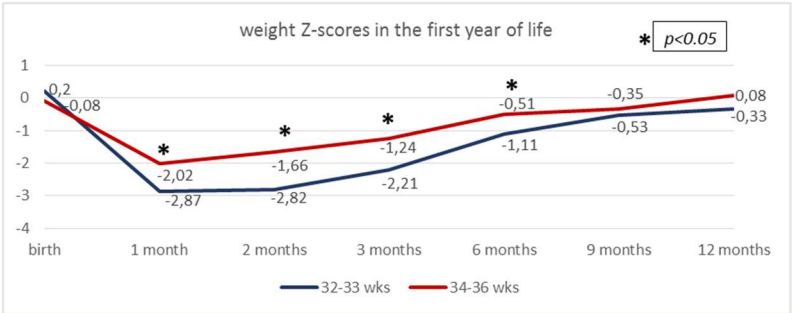
Introduction: Moderate preterm (MPT) and late preterm (LPT) represent the majority of preterm births and up to 5-9% of all live births. The growth pattern of infants born severely preterm has been correlated in recent years to their neurodevelopmental outcome, but few data are available regarding the growth of infants born at 32-36 weeks of gestational age (GA).

Materials and methods: A prospective observational study on subjects born at 32-36 weeks GA at the University Hospital of Siena in the years 2016-2017 was conducted. The aim was to evaluate the pattern of weight growth during the first year of life of MPT and LPT newborn infants. The newborns were divided into two groups: MPT ($32\text{-}33^{+6}$ wks GA n=16) and LPT ($34\text{-}36^{+6}$ wks GA, .n=39 infants). Data related to obstetric and neonatal history were collected from medical records. The auxological parameters at birth and discharge were calculated with reference to INeS neonatal anthropometric charts. All the newborns under examination were followed in a follow-up program, periodically at 1, 2, 3, 6, 9, 12 months. The z-scores (zsc) of weight were calculated with reference to the WHO 2006 growth charts.

Results: Anthropometric data at birth were appropriate for gestational age in the two groups. The need of nutritional supports at birth (parenteral nutrition, intravenous infusion, gavage) was significantly greater in MPT than LPT. From birth to discharge, 56.2% of MPTs vs. 48.7% of LPT showed a change in weight zsc>1DS.

	Moderate Preterm (n=16)			Late Preterm (n=39)		
	Weight (g)	°ct	z-score	Weight (g)	°ct	z-score
Birth	1920 (413)	56.8 (31.4)	0.20 (1.08)	2435 (439)	47 (26.9)	-0.08 (0.96)
Discharge	2475 (333)	25.9 (22.2)	-0.89 (0.92)	2482 (295)	20.1 (20.2)	-0.99 (0.0)
1 month	2890 (547)	3.6 (6.7)	-2.87 (1.21)	3315 (630)	10.7 (18.0)	-2.02 (1.31)
2 months	3676 (667)	3.0 (4.2)	-2.84 (1.20)	4378 (805)	16.9 (21.7)	-1.66 (1.42)
3 months	4612 (729)	6.7 (9.9)	-2.21 (1.14)	5309 (907)	23.8 (26.1)	-1.24 (1.36)
6 months	6620 (720)	20.1 (20.1)	-1.11 (0.86)	7202 (871)	37.2 (28.3)	-0.51 (1.06)
9 months	7973 (795)	34.0 (26.2)	-0.53 (0.82)	8298 (1060)	40.4 (29.5)	-0.35 (1.12)
12 months	8873 (881)	40.7 (27.7)	-0.33 (0.88)	9429 (1057)	52.9 (28.1)	0.08 (0.97)

A significant difference in the zsc was observed between the two groups at 1, 2, 3 and 6 months of life; this difference was no longer appreciable in the second semester of life, with both groups reaching the average of the reference population (zsc -0.33 MPT; 0.08 LPT). MPTs showed a growth retardation from birth for the first 3 months of life, with evidence of catch-up growth (reaching a DS>-2) between 3 and 6 months of life and recovery at 12 months; instead, LPTs showed a more linear weight growth trend with a gradual recovery after the first month of life.



Conclusion: Growth trajectories of the groups appeared significantly different, with MPTs showing a pattern more similar to severely-preterm infants and LPTs more similar to full-term ones. MPTs present an extrauterine growth deficit that lasts up to 3 months, but the subsequent catch-up between 3 and 6 months allows to reach the average centile of the reference population at 12 months. Further studies are needed to evaluate whether this growth rate can influence body metabolism of MPT infants in later ages.

ANNEX 7

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Original article

Personality, emotional and cognitive functions in young adults born preterm

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Abstract

Background: Survival of preterm very low birthweight infants resulted in high risk for developmental cognitive deficits, poor academic achievement, and behaviour disorders. While numerous studies evaluated the prevalence of neurodevelopmental disability in early childhood, poor literature is available for infants born very low birthweight in adulthood.

Materials and methods: Fifty-five young adults born preterm (mean age: 18 ± 2.42 years; <33 weeks of gestational age and/or with birth weight <1500 g) were enrolled. The Verbal Intelligence Quotient (vIQ), Performance Intelligence Quotient (pIQ) and Total Intelligence Quotient (tIQ) were assessed through the Wechsler Adult Intelligence Scale – Revised (WAIS-R). Personality profiles were investigated using Rorschach test. Both WAIS-R and Rorschach scores were subsequently compared to 13 matched controls born at term. Data were analysed with the SPSS v20 for Windows statistical package.

Results: Young adults born preterm showed lower IQ scores than young adults born at term: tIQ 90.95 ± 22.46 *versus* 108.77 ± 16.14, p = 0.006; vIQ 89.85 ± 21.85 *versus* 107.69 ± 18.33, p = 0.009, and pIQ 92.40 ± 22.90 *versus* 108.31 ± 14.52, p = 0.011. No differences emerged in personality profile as most subjects showed adequate internal resources in both groups, but a trend towards anxiety and insecurity were identified in young adult born preterm.

Conclusions: Young adults born preterm show psychological fragility and lower cognitive pattern than young adults born at term. Data support the need of an early psychological intervention that could help these individuals at greater risk to face a young society that is changing and that necessarily requires stronger internal resources.

Keywords: Preterm newborns; Young adult; Developmental outcome; Intelligence quotients

1. Introduction

Advances in obstetric and neonatal medical care during the last two decades paved the way to increased survival rates of very preterm and very low birth weight (VLBW) infants. Survival at the limit of viability could result in high risk for disability and developmental problems including cognitive deficits, poor academic achievement, and behavior disorders. Nevertheless, advances in perinatology have led to an improvement in the gross motor outcome with a reduced incidence of cerebral palsy [1]. Conversely, prematurity still correlates with

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a wide spectrum of late developmental abnormalities and behavioral disorders, from milder to more severe social difficulties and functional impairments [2,3].

While numerous studies evaluated the prevalence of neurodevelopmental disability in early childhood, less literature is available for infants born preterm or low birth weight (BW) in adolescence and adulthood. Moreover, even if emerging, few data are available about perception, affect, and social cognition of young adult born preterm [3].

Despite the heterogeneity in the definition of cognitive deficits, various studies report that preterm babies have greater cognitive disability than term counterparties, with significant mean differences of IQ (Intelligence Quotient) scores [3–10]. The importance of postnatal environmental factors in subsequent neurodevelopment is well described. Environmental influences, such as parenting or schooling, may lead to changes in cognitive function over time [4]. In particular, there seems to be a correlation between level of education of the primary caregiver and cognitive and language development in the first two years of life [11] and a correlation between a good early parent-infant relationship and the subsequent cognitive ability of the young adult born preterm [12].

In addition to the cognitive functioning, another remarkable issue in the transition from childhood to adolescence and adulthood is represented by the emotional and personality pattern that could influence social interactions and social risk of the young adults born preterm. Literature data are conflicting about this point: while many studies report an increased risk of mental disorders and emotional problems for adults born preterm than those born at term [3,13–15], recent studies do not confirm a higher risk for mood disorders or mental health-related problems in ex-preterms [15–17].

Considering that studies on the personality of young adults born preterm are mainly based on self-report and are limited in number, it seems interesting to deepen the relationship between prematurity and personality traits in young adults born preterm, especially in view of potential early treatments.

The present study aimed to assess personality, emotional and cognitive functions in a cohort of young adult born preterm, testing the hypothesis that these subjects have cognitive and emotional consequences that persist into adulthood, compared to a cohort of peers born at term.

2. Materials and methods

In this hospital-based follow-up study, 55 young adults born <33 weeks of gestational age and/or with BW <1500 g, admitted at birth to the NICU of Siena Hospital were recruited (n = 55; mean age: 18 ± 2.42 years). All subjects with genetic or malformative syndromes, metabolic diseases and severe disabilities (cerebral palsy, blindness or deafness) were excluded since they would not have been able to perform the Wechsler Adult Intelligence Scale.

Thirteen young adults born at term (mean age: 21 ± 2.55 years) in the same geographical area were enrolled as controls. Local Ethical Committee approved the study and written informed consent was obtained from all participants. Medical history and perinatal data were obtained by medical record, and a questionnaire on current health conditions, school career, lifestyle, family structure and socio-economic status was collected from each subject.

Neonatal variables such as BW, gestational age (GA), Apgar score at 1 and 5 min, maternal age at birth and socio-economic status based on the education and occupation of one parent, neonatal neurological diseases, days on mechanical ventilator, days of hospitalization, breastfeeding or formula feeding, including known risk factors for poor neurological outcome, were considered for the analysis.

The Verbal IQ (vIQ), Performance IQ (pIQ) and Total IQ (tIQ) were obtained through the Wechsler Adult Intelligence Scale – Revised (WAIS-R). The Rorschach and the Machover tests were used to assess personal and emotional profile. The Rorschach test has long been known to be a reliable tool to analyze both subconscious personality characteristics and emotional functioning. The Machover test is a projective test of personality, showing how the drawing of the human figure may be closely related to the impulses, anxieties and conflicts of the individual.

WAIS-R, Machover and Rorschach scores were attributed by an expert and certificate psychologist. Subsequently the same healthcare provider compared the results to 13 same-age cohort born at term.

Data were analyzed with the SPSS v20 for Windows statistical package (SPSS Inc., Chicago, IL, USA). Mann-Whitney's non-parametric test for independent samples with a 95% significance level was applied for descriptive data; intergroup comparison was performed using the Chi-square test or Fischer's exact test for categorical variables; *p-values* <0.05 were considered statistically significant. For IQ, Rorschach and Machover test, the correlations with clinical and sociodemographic variables were assessed through Spearman's rho, using as a factor the study group (case *versus* control). Regarding the Rorschach and Machover analysis, each population was first analyzed with one-way ANOVA and the Pearson method for correlations; afterwards the two populations were compared with the Wilcoxon rank test in order to investigate significant differences between distributions. A multivariable regression model was used to evaluate which factor most affected IQ, using the known variables for poor neurodevelopmental

outcome, such as BW, breastfeeding and parental education or marital status, as confounding factors.

3. Results

The descriptive analysis of the population is reported in Table 1, while sociodemographic data of case and control group are illustrated in Table 2.

Young adults born preterm and those born at term showed both average IQ scores within the normal range (mean: IQ score 90–109). IQ scores were significantly lower in young adults born preterm than young adults born at term: tIQ 90.9 ± 22.4 *versus* 108.7 ± 16.1, p = 0.006; vIQ 89.8 ± 21.8 *versus* 107.6 ± 18.3, p = 0.009; and pIQ 92.4 ± 22.9 *versus* 108.3 ± 14.5, p = 0.011 (Table 3).

Statistically significant correlations were found between pIQ and being born at term (r = 0.310, p = 0.010), GA (r = 0.277, p = 0.022), BW (r = 0.428, p = 0.000), Apgar score at 5 min (r = 0.296, p = 0.041), vote at secondary school (r = 0.291, p = 0.004), maternal education level (MEL) (r = 0.380, p = 0.002), married mother (r = 0.284, p = 0.031) and married father (r = 0.290, p = 0.030) (Fig. 1). Negative correlations were found between pIQ and walking age (r = -0.392, p = 0.014) and speaking age (r = -0.305, p = 0.011) (Fig. 1). Similar findings were reported also for tIQ and vIQ.

The multivariable regression model, using BW, Apgar score at 5 min, being born at term, parental education and marital status as confounding factors, showed that MEL and BW were the most important and significant factor positively affecting tIQ and pIQ (respectively for tIQ: MEL p = 0.048, B: 6.41; BW p = 0.038, B = 0.007; for pIQ: MEL p = 0.006, B: 14.97; BW p = 0.001, B = 0.011;). vIQ was significantly associated with MEL only (p = 0.011, B: 8.14).

No statistically significant differences emerged in personality profile as most subjects showed adequate internal resources in both groups (48% cases and 30% controls). However a trend towards emotional immaturity at the Rorschach test (30% cases *versus* 7% controls) and anxiety and insecurity at the Machover test were identified in the case group with regard to controls (56% *versus* 31%). Even introversion traits were more evident in the cases *versus* controls (14% *versus* 0%) (Table 4, Fig. 2).

4. Discussion

Few studies have undertaken a comprehensive assessment of general cognitive, emotional and personality outcome among young adults of VLBW infants [2,3,5]. The present study aimed to evaluate whether young adults born preterm exhibited different emotions compared to peers born at term and whether their personality was affected by prematurity.

The Authors investigated both cognitive functions and personality in a cohort of young adults born preterm in relatively recent times (years 1988–2000). Most of the long-term follow-up studies on adults born preterm concern subjects born in previous years, at the dawn of Neonatal Intensive Care Units, thus before many advances in neonatology could have influenced outcomes [3,9,17].

In the present study, all young adults showed average IQ scores within the normal range even if significant differences in cognitive functions of young adults born preterm with respect to those born at term were found. The tIQ, vIQ and pIQ were significantly lower in young adults born preterm compared to controls, with an average deviation of 17 points between the two groups. In line with current literature, prematurity and low BW were correlated to lower IQ in young adults born preterm. Neverthless the average difference of the intelligence scores between young adults born preterm and peers born at term was higher when compared to the majority of the available data [3–10]. Data suggest that,

Table 1
Perinatal data in case and control groups.

	Cases (n = 55)	Controls (n = 13)	p-value
Maternal Age (years), mean (SD)	31 (4.72)	31 (4.04)	ns
Gestational Age (weeks), mean (SD)	31 (2.72)	39 (1.44)	0.000
Birth weight (grams), mean (SD)	1414 (319.15)	3231 (381.82)	0.000
Male gender, n (%)	31 (56)	6 (46)	ns
Apgar score at 1 min, median (IR)	5 (1–10)	9 (8–10)	0.000
Apgar score at 5 min, median (IR)	8 (1–10)	10 (10–10)	0.000
Neonatal resuscitation, n (%)	47 (87)	0 (0)	0.000
Intraventricular Heamorhage, n (%)	19 (34)	–	–
Intraventricular Heamorhage, high grade (≥grade III), n (%)	4 (7)	–	–
Periventricular Leukomalacia, all grade, n (%)	2 (4)	–	–
Hospital stay (months), mean (SD)	2 (1.11)	–	–
Mechanical ventilation (days), mean (SD)	8 (13.30)	–	–
Breastfeeding rate, n (%)	14 (25)	10 (76)	0.001

Table 2
Sociodemographic data in case and control groups.

	Cases (n = 55)	Controls (n = 13)	p-value
Age at assessment (years), mean (SD)	18 (2.42)	21 (2.55)	ns
Caucasian population, n (%)	53 (98)	13 (100)	ns
Same region of residency, n (%)	52 (94)	12 (92)	ns
Walking age (gestational age corrected months), mean (SD)	15 (3.10)	12 (2.40)	0,000
Speaking age (gestational age corrected months), mean (SD)	14 (4.34)	11 (3.15)	0,042
Good performance at elementary school (vote ≥7/10), n (%)	26 (78)	13 (100)	0,000
Good performance at secondary school (vote ≥7/10), n (%)	30 (83)	13 (100)	0,000
Mother's educational level (≥Upper secondary school), n (%)	30 (55)	12 (92)	0,019
Maternal occupation (managerial job), n (%)	8 (17)	3 (25)	ns
Father's educational level (≥Upper secondary school), n (%)	28 (53)	10 (76)	ns
Paternal occupation (Managerial job), n (%)	7 (16)	6 (50)	0,016
Marital status (Conjugated), n (%)	36 (78)	10 (83)	ns

Table 3
Wechsler Adult Intelligence Scale – Revised scores in case and control groups.

	Cases (n = 55)	Controls (n = 13)	p-value
Total Intelligent Quotient, mean (SD)	90.95 (22.46)	108.77 (16.14)	0.006
Verbal Intelligent Quotient, mean (SD)	89.85 (21.85)	107.69 (18.33)	0.009
Performance Intelligent Quotient, mean (SD)	92.40 (22.90)	108.31 (14.52)	0.011

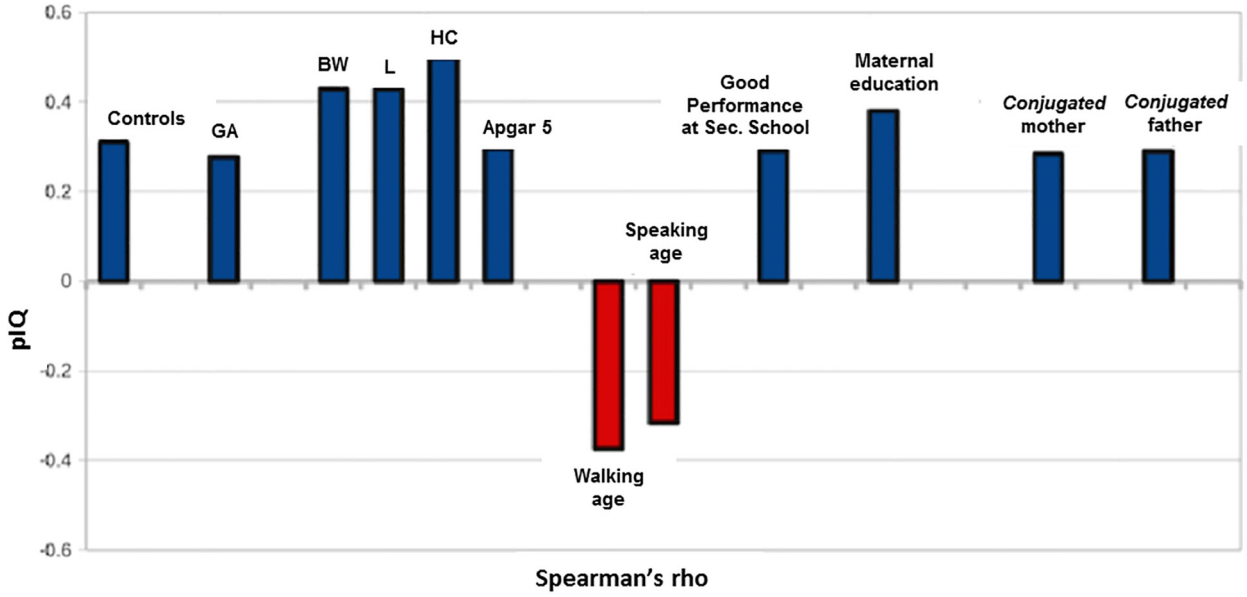

Fig. 1. Non-parametric correlations (Spearman's rho) for young adults born preterm (pIQ: Performance Intelligent Quotient; GA: gestational age; BW: birth weight: L: length: HC: head circumference).

even in the absence of severe neonatal and especially neurological problems, preterm birth exerts significant and long-term effects on the developing brain.

Negative correlations were found between pIQ and walking/ speaking age, which were significantly higher in young adult born preterm compared with controls. Positive correlations were found between pIQ and GA, BW, Apgar score at 5 min, good performance at secondary school and MEL. In fact, literature reports a positive effect of a high level of parental education on child cognitive development [11,12], remarking the importance of the interrelation between environmental context, neuromotor development in the first phases of life, and subsequent cognitive functioning.

Preterm infants, lying in their incubator separated from their parents, are often exposed to many stressful stimuli per day, aiming to prevent, diagnose or treat life-threatening conditions. The increased vulnerability

Table 4
Rorschach test and Machover test in case and control groups.

	Cases n (%)	Controls n (%)	p-value
Rorschach test			
Appropriate performance	26 (48)	5 (30)	ns
Modest performance	8 (15)	5 (48)	ns
Emotional immaturity	16 (30)	1 (7)	ns
Depressive traits	4 (7)	2 (15)	ns
Machover test			
Appropriate behaviour	14 (26)	8 (61)	ns
Introversion	7 (14)	0 (0)	ns
Anxiety and insecurity	29 (56)	4 (31)	ns
Relationship problems	2 (4)	1 (8)	ns

to pain and the immaturity of the nociceptive modulation pathways could impact the developing brain during this vulnerable period [18]. In preterm children without major sensory, motor or cognitive impairments a significant association between neonatal pain-related stress and thinner cortex in multiple regions at school age has been reported [19].

Despite neonatal non-modifiable risk factors, a good family socioeconomic status and a high level of parental education may positively influence the global child development.

Rorschach and Machover projective tests have been used in order to study the emotional pattern, since they are considered to be related to the impulses, anxieties and conflicts of the person; in particular, the Rorschach test is useful to analyze the subconscious personality characteristics and emotional functioning [20]. The majority of young adults born preterm showed appropriate internal resources, although notes of emotional immaturity, anxiety and emotional stress were found to a greater extent in the case group. It is plausible that the small sample size of the cohort of young adults born preterm results in limited power to detect a significant relationship between prematurity and emotional disorders. However, the described dysfunctional features found in personality and emotional functioning strongly support the hypothesis that young adults born preterm are at increased social risk. This finding could be related to maternal anxiety, indepen-

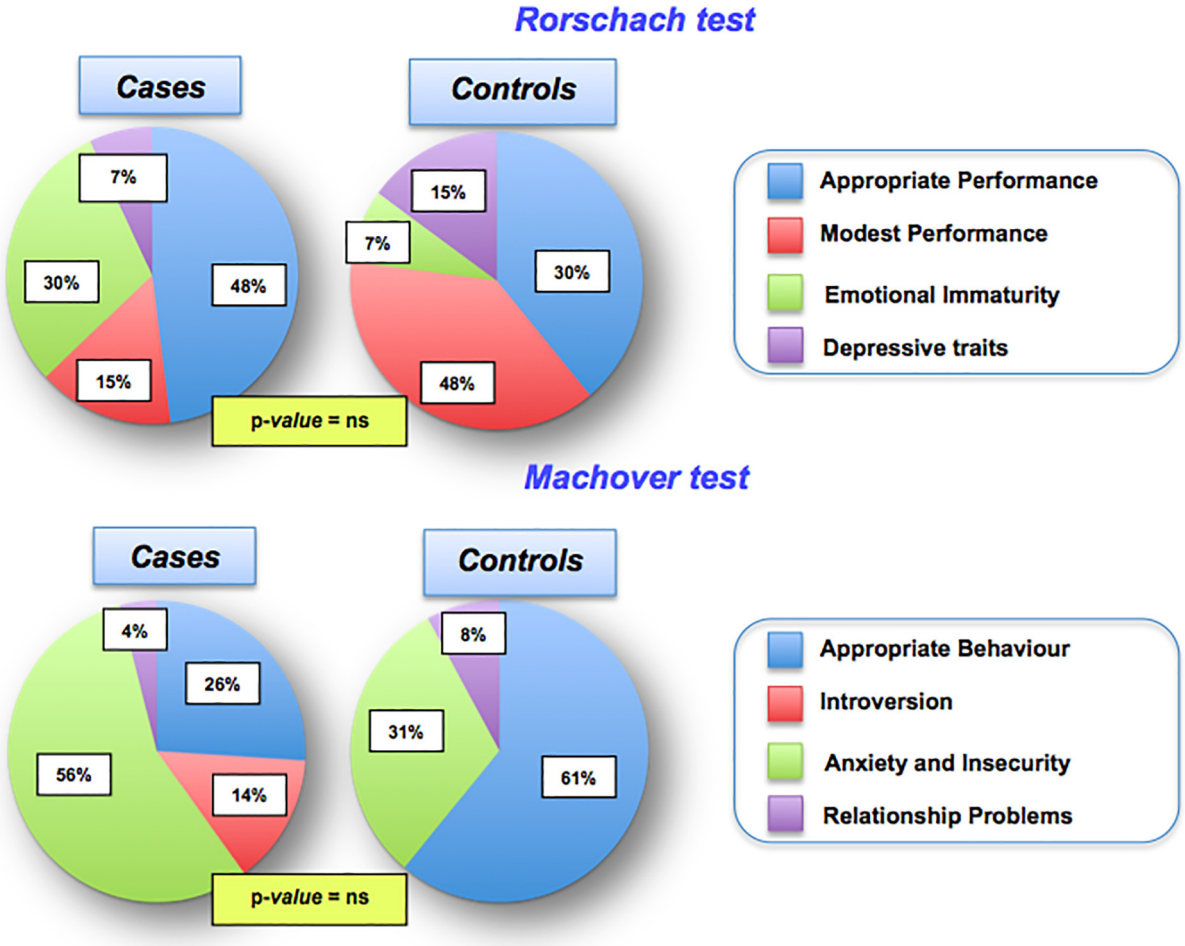

Fig. 2. Intergroup results for the Rorschach test and the Machover test.

dently of social and perinatal factors as previously described [21]. Maternal feelings like frustration and perception of inadequacy persisting beyond the perinatal period may have a negative impact on the mother–child interaction that in turn affects the emotional, behavioural and cognitive outcomes of children born preterm. At the time of our study, parent's access into the NICU was possible only for a few hours a day leading to a possible separation experience. Nowadays the family-centered developmental care with parent access 24 h per day, the promotion of a continuous mother–child contact together with a more carefulness in everyday NICU practices, procedures and physical environment are considered crucial to the physical, emotional and social well-being of both infant and parent [22–24]. All together, the reported results contribute to deepen the likely multifactorial origin of behavioural, emotional and cognitive problems in young adult born preterm, which could result from the interaction of perinatal, neurological, and environmental factors [25]. The results also encourage to implement non-pharmacological strategies to reduce pain/stress in the NICUs and to positively stimulate brain development, possibly by enriching the protective environment in NICUs. Individualized developmental care techniques such as kangaroo care, promotion of sleep, massage, mothers voice, and music could determine an improvement in neonatal neurodevelopment, through comfort, pain reduction or even the stimulation of experience-dependent neural circuits. [22,26–28].

This study has the limitation of including a small number of term control young adult and this may have affected the results for the personal profiles. Further research is needed in this field to confirm our data.

In conclusion, young adults born preterm show psychological fragility. Behavioral assessment may reveal peculiar personality characteristics in young adult born preterm that could benefit of early intervention. Full-scale IQ and behavioral and emotional assessment should be considered both as a crucial part of follow-up programs. Intervention projects designed to support parents and promote parents-to-child relationship from the first years of life to adolescent period could allow a better social integration and the chances of permanent employment as adults. An early psychological intervention could help these individuals at greater risk to face a young society that is changing and that necessarily requires stronger internal resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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ANNEX 8

Perrone S., Laschi E., Buonocore G. *Biomarkers of oxidative stress in the fetus and in the newborn.* Free Radic Biol Med. 2019 Oct; 142:23-31. doi: 10.1016/j.freeradbiomed.2019.03.034.

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Review Article

Biomarkers of oxidative stress in the fetus and in the newborn

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ARTICLE INFO

Keywords:
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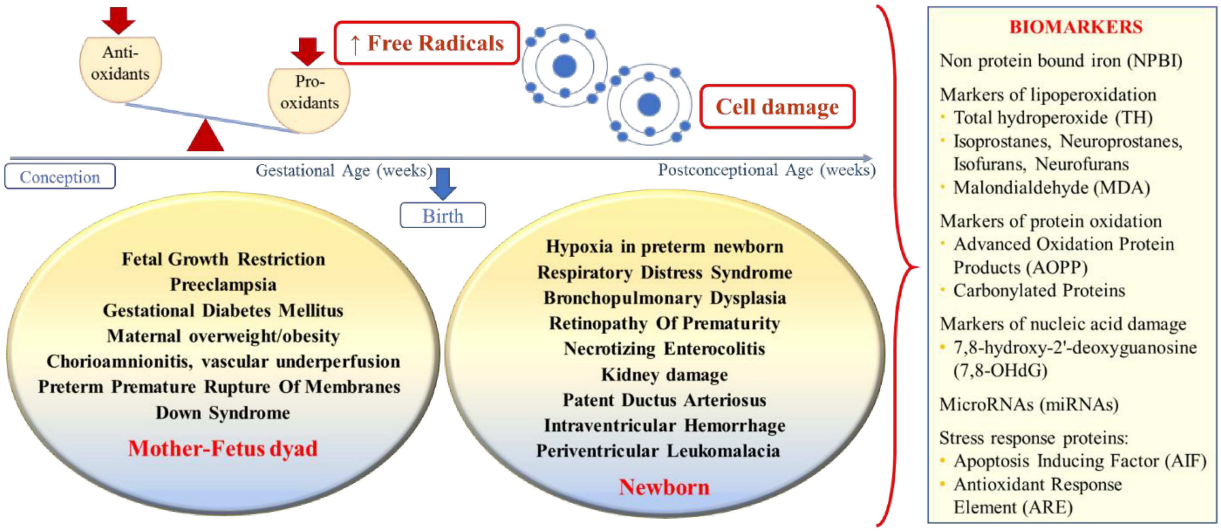
Isoprostanes
Free radicals
Fetus
Newborn infants

ABSTRACT

The dynamic field of perinatology entails ever-increasing search for molecular mechanisms of neonatal diseases, especially in the domain of fetal growth and neurodevelopmental outcome. There is an urgent need for new molecular biomarkers, to early identify newborn at high risk for developing diseases and to provide new treatment targets. The interest in biomarkers of oxidative stress in perinatal period have begun to grow in the last century, when it was evidenced the importance of the free radicals generation underlying the various disease conditions. To date, interesting researches have been carried out, representing milestones for implementation of oxidative stress biomarkers in perinatal medicine. Use of a panel of "oxidative stress biomarkers", particularly non protein bound iron, advanced oxidative protein products and isoprostanes, may provide valuable information regarding functional pathways underlying free radical mediated diseases of newborns and their early identification and prevention.

Here, we will review recent advances and the current knowledge on the application of biomarkers of oxidative stress in neonatal/perinatal medicine including novel biomarker discovery, defining yet unrecognized biologic therapeutic targets, and linking of oxidative stress biomarkers to relevant standard indices and long-term outcomes.



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Here, we will review recent advances and the current knowledge on the application of biomarkers of oxidative stress in neonatal/perinatal medicine including novel biomarker discovery, defining yet unrecognized biologic therapeutic targets, and linking of oxidative stress biomarkers to relevant standard indices and long-term outcomes.

1. Oxidative stress and biomarkers

Perinatal oxidative stress (OS) is an unavoidable consequence of the life in an oxygen-rich atmosphere. OS occurs when the burden of free radicals (FRs) production is not adequately counteracted by the intracellular antioxidant systems. Foetuses and newborns are highly prone to the oxidative insult, due to the overloading of aerobic metabolism linked to the rapidly growing energy demand, the presence of conditions leading to excessive FRs production, the presence of high non protein-bound iron (NPBI) levels, and the immaturity of antioxidant systems. FRs are generated by endogenous and exogenous mechanisms such hypoxia, asphyxia, ischemia, ischemia-reperfusion, hyperoxia, inflammation, mitochondrial impairment, increased free circulating transition metals environmental agents and xenobiotics [1.]. The maintenance of the balance between pro-oxidants and anti-oxidants is critical to the normal cellular functions. The dangerous effects of FRs are related to both their property of being very unstable molecules and their ability to react with polyunsaturated fatty acids of cell membranes, proteins, polysaccharides, nucleic acids, causing functional alterations within the cell. OS is largely responsible of cellular, tissue and organ damage in the perinatal period. It is also strongly involved in fetal programming of adult diseases, through the gene expression regulation and cell growth modulation [2.]. Maternal diabetes, prenatal hypoxic/ischaemic events, inflammatory/infective insults are specific triggers for an acute increase in free radicals generation. Oxidative stress may be the general underlying mechanism that links altered placental function to fetal programming.

There is a continuing and growing need to develop a panel of biomarkers that could allow detecting diseases and treatment responses.

According to the definition of the Food and Drug Administration (FDA)/National Institutes of Health (NIH), a biomarker is "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions" [3.]. Biomarkers of disease activity would be involved in the disease process or released because of tissue damage during disease progression. The identification of reliable biomarkers is essential for the characterization of OS and probably for the early discovery of OS-associated diseases. Biomarkers evaluate host susceptibility to OS by measuring proteins, lipids and DNA damage. They are useful for monitoring pharmacologic response to antioxidant interventions. They can be used as "intermediate endpoints or early-outcome predictors" of disease development and for preventive purposes [4.], [5.]. A complete and reliable biomarker should have biological validity, high sensibility and specificity, a

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Abbreviations

7,8-OHdG	7,8-hydroxy-2′deoxyguanosine
AOPP	advanced oxidative protein products
BPD	bronchopulmonary dysplasia
CA	chorioamnionitis
DHA	docosahexaenoic acid
F2-IsoP	F2-isoprostanes
F4-NPs	F4-neuroprostanes
FGR	fetal growth restriction
FRD	free radicals disease
FRs	free radicals
GDM	gestational diabetes mellitus
HIE	hypoxic-ischemic encephalopathy
IUGR	intrauterine growth restriction
IVH	intraventricular hemorrhage
MDA	malondialdehyde
MiRNAs	microRNAs
NEC	necrotizing enterocolitis
NFs	neurofurans
NPBI	non protein bound iron
$^{\circ}OH$	hydroxyl radical
OS	oxidative stress
OSI	oxidative stress index
PDA	patent ductus arteriosus
pPROM	preterm premature rupture of membranes
PVL	periventricular leukomalacia
RDS	respiratory distress syndrome
RNS	reactive nitrogen species
ROP	retinopathy of prematurity
ROS	reactive oxygen species
TH	total hydroperoxides
TAC	total antioxidant capacity
TOS	total oxidant status
VU	vascular underperfusion

standardized methodology of measurement and should be reproducible.

OS is difficult to be measured in vivo, because oxygen-centered radicals (reactive oxygen species, ROS), nitrogen-centered radicals (reactive nitrogen species, RNS) and FRs have a very short half-life. General analytical methods available to study oxidative injury can be divided into two categories: those aimed at detecting potential risk of oxidative stress, such as non protein bound iron due to its capacity to generate hydroxyl radical ($^{\circ}OH$) through Fenton reaction, and those aimed at detecting oxidation in lipids, proteins and DNA. Stress response proteins such as apoptosis inducing factor (AIF) [6] and antioxidant response element (ARE) [7], can also be used as OS biomarkers. Disturbances of synthesis, expression and activity of these proteins may be inherited, and they are under modification risk by OS. Because of the multiples effects of OS, in most studies there is not described a single biomarker related to a singular disease or condition, but rather a panel of biomarkers that together can allow detecting a risk condition or a fetal-neonatal pathology.

1.1. Non protein-bound iron (NPBI)

Iron, a highly reactive element, is one critical component for the generation of free radicals being a strong biologic oxidant and a reducing agent. In particular, iron catalyses the formation of the highly reactive $^{\circ}OH$ from hydrogen peroxide in the Fenton reaction: $Fe^{2+} + H_2O_2 > Fe^{3+} + {}^{\circ}OH + OH^{-}$.

In moderate quantities and bound to protein, it is an essential element for growth and for all aerobic metabolic processes, but it is toxic when unbound. Physiologically, iron is safely sequestered in transport proteins such as transferrin and lactoferrin, and stored in proteins like ferritin and hemosiderin. Iron ions cannot exist in plasma, so the term non protein-bound iron (NPBI) was introduced to indicate a low molecular mass iron form, free from the high-affinity binding to transferrin. In this case, iron is available to react with reduced intermediates of oxygen and generate reactive oxygen species (ROS). These FRs are capable of releasing even more iron by mobilizing it from ferritin [8]. Therefore, the toxicity of iron is inversely proportional to the availability of ferritin necessary for sequestering and detoxifying ferrous ion, and directly proportional to the quantity of hydrogen peroxide available for producing hydroxyl radicals by the Fenton reaction [9.].

Erythrocytes have been the first cells to reveal the neonatal susceptibility to oxidative stress. OS leads to the oxidation of haemoglobin and to the damage of the erythrocytes membranes [10.]. Membrane structure alterations and modification of erythrocytes functions following OS have been studied by not only incubating red cells with oxidizing agent such as phenyl-hydrazine, but also incubating them under anaerobic and aerobic conditions [11.]. Asphyxia and acidosis supply redox-cycling iron, predisposing the increase of the free iron content of erythrocytes. Free iron release is accompanied by the oxidation of membrane proteins and the appearance of senescent cell antigen, one of the major pathways for erythrocyte removal, as measured by autologous IgG binding [12.]. In newborns, the release of NBPI in erythrocytes correlates with plasma NBPI: the released iron has a tendency to diffuse from erythrocytes into the surrounding medium, suggesting the appearance of plasma NBPI [13.]. In hypoxic newborns, increased concentrations of plasma NBPI significantly correlate with the severity of the brain injury and alteration of neurodevelopmental outcome until the second year of age [14.]. Plasma free iron seems to be a reliable index of brain damage, reaching 100% sensitivity and specificity at high concentration. The significant positive correlation between plasma free iron and the number of nucleated red cells in cord blood, currently considered a reliable index of lasting intrauterine asphyxia, suggests that the rate of erythropoiesis and the entity of NPBI are related to the degree of asphyxia and to the probability of subsequent neurological impairment [15.]. Moreover, a supposed interrelation between NPBI and white matter injury in preterm hypoxic newborns has been advanced. Supportive of a relationship between iron and periventricular leukomalacia (PVL) is the observation that, many weeks after human intraventricular hemorrhage and post-hemorrhagic ventricular dilatation (disorders that sharply increases the risk of PVL), levels of non-protein-bound iron in the cerebrospinal fluid are markedly increased [16.]; this increase could not be explained by hemolysis alone. Furthermore, increased release of free iron has been demonstrated in brain structures of neonatal animals after asphyxia [17.].

1.2. Lipid peroxidation: isoprostanes, isofurans, neuroprostanes, neurofurans; malondialdehyde

In the presence of a free radical or a free radical initiator biological membranes, which contain a relatively high proportion of polyunsaturated lipids, become susceptible to oxidation. Free radical-induced peroxidative damage to membrane lipids is an event potentially leading to critical cell injury. If not interrupted, in certain conditions it may cause an irreversible damage to the cells and may initiate or promote the pathogenesis of injury or disease state.

Lipid peroxidation provides a number of possibilities for assays. It is a radical process whereby polyunsaturated fatty acid (PUFA) contained in the phospholipids of cellular membranes undergo a reaction with oxygen, yielding lipid hydroperoxides. The reaction occurs through a free radical chain mechanism initiated by the abstraction of a hydrogen atom from PUFA by a reactive free radical, followed by a complex

sequence of propagative reactions. Hydroperoxides are the major initial molecular products of lipid peroxidation and can be measured in plasma by a variety of techniques. *Total hydroperoxide (TH)* represents a measure of overall OS, because it is indicative of intermediate oxidative products of lipids and peptides. Lipid and protein damage from FRs exposure leads to lipid hydroperoxide generation from lipids and to carbonyl formation and protein hydroperoxide generation from proteins. Lipid and protein hydroperoxide, in the presence of traces of free iron, produces several secondary reactive radical species, which can be measured collectively as organic hydroperoxide. Because of the rapid degradation in vitro, an accurate measurement of hydroperoxides is very difficult. The fact that the origin of lipid peroxidation products cannot be directly demonstrated represents a significant problem with the lipoperoxidation tests. This limitation can be overcome by measuring a series of prostaglandin-like compounds, called isoprostanes (IsoPs) and isofurans (IsoFs). Isoprostanes and isofurans are produced independently of the cyclooxygenase pathway, and their formation results respectively from oxidation of arachidonic acid (AA) and docosahexaenoic acid (DHA). The *F2-isoprostanes* are prostaglandin-like products, which originate from in vivo and in vitro peroxidation of arachidonic acid and phospholipids. They are not produced by cyclooxygenase but just by free radicals reactions. F2-isoprostanes are initially formed in phospholipids and then released into the blood. These prostanoids are less reactive and unstable than other peroxidation products such as aldehydes or peroxyl radicals, so they can be easily measured out in plasma and urine using methods such as the gas chromatography coupled to mass spectrometry (MS) technique, liquid chromatography coupled to MS (LC-MS) and immunological assays [9.]. Normal adult humans are found to have stable plasma levels of F2-isoprostanes. When compared with adults, the plasma F2-IsoP levels of newborns are significantly higher and an inverse relationship between IsoPs levels and gestational age was reported [18.], suggesting that lipid peroxidation is already active in the antenatal period and that it goes to fade during the last gestational weeks and throughout postnatal life. A recent study made it possible to determine the F2-IsoPs reference levels in newborns [19.]. An oxygen insertion step diverts intermediates from the IsoPs pathway to form other compounds, termed *isofurans (IsoFs)* that contain a substituted tetrahydrofuran ring. Because of this differential method of formation, it has been underlined that oxygen tension can affect lipid peroxidation profile [20]. Like the IsoPs, the IsoFs are chemically stable so can act as in vivo biomarkers of oxidative damage. Moreover, the ratio of IsoFs/IsoPs provides information about the relative oxygen tension where the lipid peroxidation is occurring.

Docosahexaenoic acid (DHA) is a major component in neuronal membranes, whose levels in the brain increase during development [21.] and decrease with aging [22.]. DHA oxidizes both in vitro and in vivo to form F2- IsoP-like compounds termed *F4-neuroprostanes* (*F4-NPs*). The NPs are in vivo-biomarkers of oxidative damage selective for neurons. An alternative pathway of oxidation of DHA bring to the formation of IsoFs-like compounds termed *neurofurans* (*NFs*). Quantitative assessment of NFs in vivo reveals modulated formation under conditions of increased or decreased OS. Epidemiological studies have linked low plasma and blood cells DHA to increased risk of poor neural development in infants and children [23.] [24.], and the integral role of DHA in membrane lipids raise the possibility that inadequate DHA alters brain development through effects on membrane structures. Given the abundance of DHA in the brain, analysis of NFs may have particular value in the quantitative assessment of lipid peroxidation in brain damage [25.].

Malondialdehyde (*MDA*) is another biomarker of lipid peroxidation, frequently used in clinical studies. MDA is one of the principal and most studied low-molecular-weight end products, highly cytotoxic because of its ability to bind proteins or nucleic acids very quickly. The thiobarbituric acid reactive substance method (TBAR test) has been frequently used to assess MDA concentrations, but it lacks specificity. There is a risk of underestimating lipid peroxidation since MDA can in vivo form Schiff bases or crosslinked bonds with lysine and arginine from proteins, so poor quantification sensitivity and poor molecular specificity can be attributed to this method. MDA is a minor secondary oxidation product; in addition, MDA is not exclusively derived from polyunsaturated fatty acids, so the interpretation of the MDA content and the TBA test response in studies of lipid peroxidation requires caution [26]. Other analytical techniques such as specific derivatization before liquid chromatography with UV or mass spectrometry detection have been proposed. Cipierre et al. proposed the malondialdehyde adduct to haemoglobin (MDA-Hb) measured in red blood cells to assess OS in preterm neonates [27.].

1.3. Protein oxidation: carbonyls and AOPP

Oxidative damage to proteins is problematic due to the large number of different protein targets and the relatively high number of different amino-acidic residues. Reactive free radicals can modify amino acid residues of proteins and lead to cross-linking, changes in conformation and loss of function. Oxidative damaged proteins are likely to be removed rapidly by proteases rather than accumulate to readily detectable levels.

1.3.1. Carbonylated proteins

During the oxidation of proteins, carbonyl groups (—CO═O) are introduced into the side-chains of the proteins. Indeed, when proteins react with hydroxyl radical there is an abstraction of a hydrogen atom from protein polypeptide, forming a carbon-centered radical, which readily reacts with dioxygen to form peroxyl radicals under aerobic conditions (Fig. 1).

The side chains of all amino acid residues of proteins are susceptible to oxidation by ROS action. In particular, lysine and arginine are involved in this “carbonyl” stress, leading to the formation of AGEs (Advanced Glycated End Product) such as pentosidine, a group of heterogeneous molecules deriving from non-enzymatic reactions of reducing sugars with amino groups of lipids, DNA, and especially proteins [28]. In the presence of transition metals, an oxidative cleavage, a loss of histidine residues, bi-tyrosine cross-links, an introduction of carbonyl groups and the formation of alkyl, alkoxyl and alkylperoxy radicals are carried out [29.]. Iron (Fe II) can bind proteins to specific sites, and the Fe II-protein complex reacts with H_2O_2 via Fenton reaction to provide ROS. The concentration of carbonyl groups, generated by many different mechanisms, is a good measure of ROS-mediated protein oxidation because of their relative early formation and their relative stability. Many assays are available to detect protein carbonyls; highly sensitive methods involve derivatization of the carbonyl group with 2,4-dinitrophenyl-hydrazine (DNPH), which leads to formation of a stable dinitrophenyl-hydrazone product easily detectable with various methods (spectrophotometric assay, enzyme-linked immunosorbent assay-ELISA, and one-dimensional or two-dimensional electrophoresis followed by Western blot immunoassay) [30.], [31.].

An increase of carbonyl groups has been demonstrated in the earliest 6 h after the advent of hypoxia-ischemia in animal models [32.]. Moreover, albumin carbonylation has been demonstrated in newborns with high NPBI levels and poor neurodevelopmental outcome [29.]. The altered protein molecules act as a trap for FRs, which start further chain reactions worsening the brain damage.

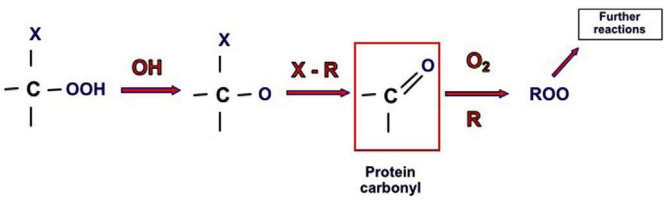

Fig. 1. Schematic representation of protein oxidation.

1.3.2. Advanced oxidation protein products

As plasma proteins are the first target of free radicals, the detection of advanced oxidation protein products (AOPP) in biologic fluids can be an optimal strategy to detect and to estimate the degree of oxidant-mediated protein damage. Indeed AOPP are terminal products of protein exposure to FRs without oxidant properties. AOPP can be measured using spectrophotometry on a microplate reader as described by Witko-Sarsat and colleagues [33.], so they represent a marker of the degree of protein damage in OS. However, current AOPP methods suffer from poor reproducibility due to precipitation of lipids in plasma samples. Hanasand et al. have proposed a novel method that provides solubilization of lipids before spectrophotometric measurement of AOPP levels, in order to prevent both loss of lipoproteins due to precipitation and overestimation because of light scattering [34]. AOPP levels are elevated in hypoxic newborns, especially preterm [35.], [36.].

1.4. Oxidative DNA damage: 7,8-hydroxy-2′-deoxyguanosine

7,8-hydroxy-2′-deoxyguanosine (7,8-OHdG) is a reliable and frequently used marker of oxidative stress and especially of OS-related DNA damage, because it is an oxidized nucleoside released upon repair of damaged DNA. 7,8-OHdG can be detected in human tissues or blood samples. Because oxidative DNA lesions like oxidized nucleosides and bases, are reasonably water-soluble and excreted into the urine without being further metabolized, urinary 7,8-OHdG is considered an important biomarker of generalized and cellular oxidative stress [37.]. This biomarker can also be detected in human peripheral leukocytes, by an ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method [38].

1.5. A novel biomarker: microRNAs

MicroRNAs (miRNAs) are a group of small noncoding RNAs, known to perform a unique role in posttranscriptional gene regulation and important in the regulation and modulation of physiological and pathological processes, even cellular responses to redox imbalance. MiR-200 family members play a crucial role in OS-dependent endothelial dysfunction, as well as in vascular complications of diabetes and obesity; in addition, different miRNAs, such as miR-210, have been shown to play a key role in mitochondrial metabolism, therefore modulating ROS production and sensitivity. Recent studies have revealed that a number of miRNAs act on placental development. Several miRNAs have been reported to be specifically expressed in the placenta, plausibly affecting OS damaging responses [39]. Taking into account this premise, circulating microRNAs in maternal blood may be potential biomarkers for fetal hypoxia in-utero and therefore useful for the detection of hypoxia in the intrapartum period [40].

1.6. OS in pregnancy and its role on the placental-fetal unit

A fragile redox balance must exist to allow a proper growth and development in pregnancy. A properly controlled oxidative species production has been proven to be physiologically a necessary factor [41.]. From the beginning of pregnancy, OS and placentation are closely interrelated, and ROS/RNS are shown to influence placenta development while conversely abnormal placentation may lead to OS and adverse consequences [42.]. Although OS is a necessary feature of normal pregnancy and normal fetal development, increased OS can originate different diseases and conditions and it can been demonstrated through OS-biomarkers concentration in biological fluids and tissues. Early in utero life is vulnerable to redox perturbation and the fetal period of development is extremely sensitive to environmental cues. Permanent structural and physiological changes may lead to long-lasting consequences in postnatal life [2.] (Fig. 2).

One of the most studied complications of pregnancy is the *Intrauterine Growth Retardation (IUGR)* or *Fetal Growth Restriction (FGR)*. By measuring IsoPs, it has be found that OS occurs early in pregnancy and can be detected in amniotic fluid of high risk pregnancies with intrauterine growth retardation, so the assay of F2- isoprostanes in amniotic fluid is a reliable assessment of fetal oxidative stress [43.]. Indeed, fetal growth restriction is related with placental insufficiency, impaired blood flow to the fetus and intrauterine hypoxia; chronic restrictions in uterine blood flow elicits placental and fetal responses in the form of growth adaptation to hypoxia, so intrauterine hypoxia may induce FRs generation and fetal OS.

Because of the lack of fetal but also maternal [44.] antioxidant systems, an excess production in ROS during the intrauterine period leads to a pro-oxidative status compromising fetal growth [45.]. Also other OS biomarkers, including malondialdehyde (MDA), have been detected increased in maternal plasma, umbilical cord plasma, and placental tissues of patients with IUGR when compared to the control group without IUGR, confirming the role of OS in the altered fetal growth [46.], [47.].

Preeclampsia (PE) is a potentially life-threatening complication of pregnancy, characterized by maternal hypertension, proteinuria, possible multi-organ failure and death; it is a condition strictly related to

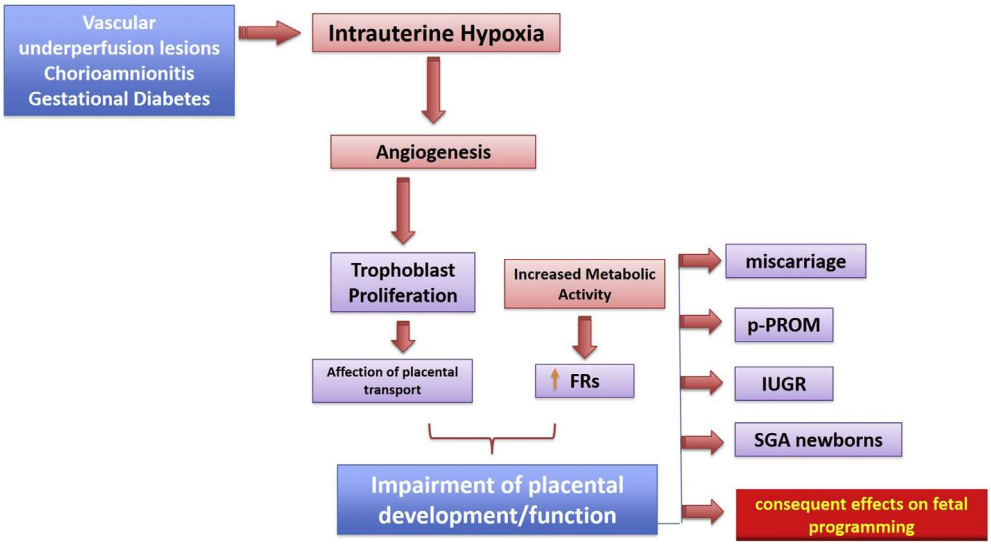

Fig. 2. Intrauterine environment and redox influence on placental function.

and cause of FGR. A complex interrelation between OS, systemic inflammation and vascular dysfunction underlies the condition: higher superoxide concentrations (as a measure of systemic OS) and markers of systemic inflammation have been found in pre-eclamptic pregnancies compared with healthy pregnancies [48.]. There is irrefutable evidence of placental OS in cases of early onset pre-eclampsia, including increased concentrations of protein carbonyls, lipid peroxides, nitrotyrosine residues and DNA oxidation [49.] [50.], [51.]. Free F2-IsoPs were found to be significantly higher in preeclamptic women compared to normotensive controls, both in placenta [52.] and in maternal plasma samples [53.]. Moreover, placental oxidative DNA damage has been related to pre-eclamptic pregnancies with fetal growth restriction, as demonstrated by the increased levels of 7,8-OHdG in placental trophoblast cells [54.], [55.].

Another frequent pregnancy complication is *Gestational diabetes mellitus (GDM),* which is known to be associated with an overproduction of reactive oxygen species and oxidative stress. Numerous evidences revealed increased biomarkers of lipid peroxidation such as MDA levels in the plasma [56.] and isoprostanes in placenta [57.] of diabetic women during pregnancy. Recent studies have confirmed the higher pro-oxidant status of women with GDM by dosing elevated levels of maternal, cord and placental MDA xanthine oxidase and 8-isoprostane, and their lower anti-oxidant defenses when compared to women without GDM [58.], [59.]. In contrast with these results, Ramírez-Emiliano J. and colleagues [60.] have recently found peroxidation lipid levels (quantified with thiobarbituric acid-reactive substances-TBARS) and carbonyl levels lower in placentas with GDM than those quantified in normal placentas. These data seem to suggest that placentas with GDM are more protected against oxidative damage, but the Authors admit that probably the difference is in the fact that their patients had better metabolic control respect to the patients of previous study on GDM and OS. These results, albeit conflicting with the previous reports, are relevant because suggest that a good dietary control may be important to prevent the increased oxidative damage caused by GDM.

A similar concept could be applicable and desirable in cases of maternal overweight or obesity, because an increased OS characterizes both obesity and gestation. Indeed, increased levels of F2-IsoPs in small-for- gestational age (SGA) newborns born by *overweight or obese mothers* have been demonstrated, underlining the need to protect pregnancy and newborns from OS related to cell and tissue damage [61.]. Malti N. et al. found high levels of maternal MDA, carbonyl proteins, nitric oxide and superoxide anion and reduced antioxidant defences in obesity; in the placenta and in the newborns of these obese mothers, they found variations of redox balance indicating high oxidative stress [62.]. As obesity in women is a rising public-health problem and FGR (a possible complication of maternal obesity) is strongly associated with the metabolic syndrome in adults, a more careful pre- and perinatal monitoring is now worthwhile [63.], [64.]. However, the usefulness of some potential interventions, such as diet supplemented with polyunsaturated fatty acids or antioxidants remains to be investigated.

Increased levels of OS biomarkers have been detected in other conditions related to an altered placental functioning, such as *chorioamnionitis (CA)* or *vascular underperfusion (VU)* associated with preterm labor: recently, we found significantly increased levels of IsoPs, NPBI and AOPP in blood cord in the CA and VU preterm cases [65.]. This finding confirms the complex interrelation between OS and the correct functioning of the fetal-placental unit, with inevitable repercussions on the newborn and the future health status according to the fetal programming hypothesis.

F2-IsoPs concentrations have been found significantly higher into the amniotic fluid in pregnancies with *preterm premature rupture of membranes (pPROM)* than in normal ones [66.], highlighting the importance of ROS- induced damage to amniotic epithelium and chorioamniotic collagen. ROS may disrupt amino acid binding in proteins and polyunsaturated fatty acids of the membrane lipid bilayers, causing cell dysfunction, modification of chorioamniotic biology and predisposing pregnancies to premature rupture of membranes. Kwiatkowski S. et al. confirmed these results, detecting increased levels of F2-IsoPs in amniotic fluid and maternal plasma in cases of pPROM [67.].

Interesting, a significant rise in OS biomarkers and in particular IsoPs levels was also found in amniotic fluid of pregnancies with fetuses suffering from *Down syndrome*, with a nine-fold increase in isoprostanes concentrations compared to normal fetuses but also greater concentrations than growth-restricted fetuses. In these fetuses, enhanced OS and lipid peroxidation occur as consequence of impaired metabolism of oxygen FRs with imbalance between the formation and removal of oxidants, specifically increased superoxide dismutase 1 (SOD-1) activity not compensated by a rise in glutathione peroxidase [68.].

1.7. OS in the newborn

Newborns and especially preterm infants are particularly vulnerable to the oxidative damage because of their major production of FRs and

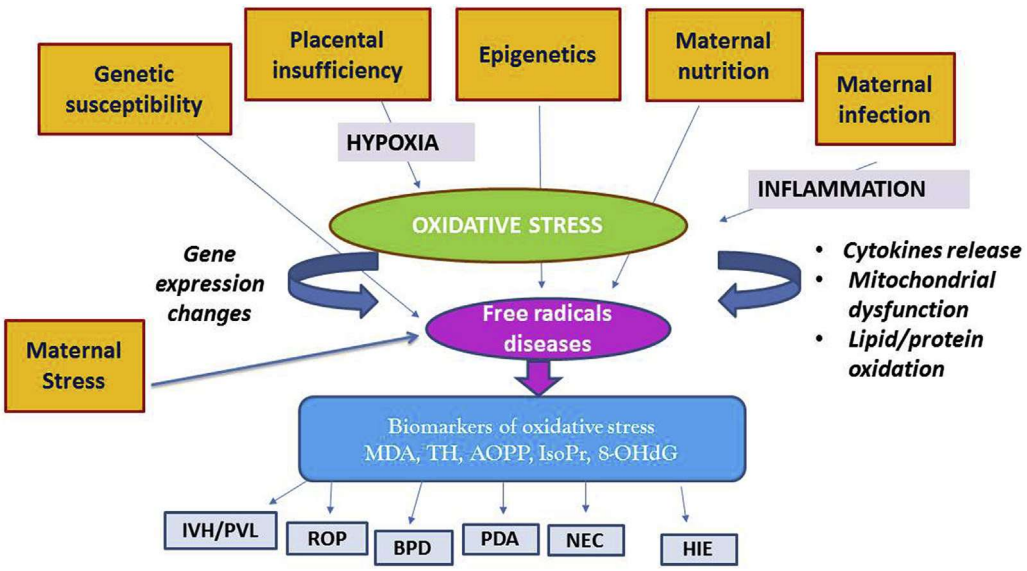

Fig. 3. The central role of OS in the pathogenesis of neonatal diseases.

their weak anti-oxidant systems with lower possibility to scavenge FRs in excess. FRs production in the postnatal period is the consequence of various biochemical events like hypoxia, hyperoxia, ischemia and re-perfusion, inflammation; all these events take place in an immature background influenced by genetic/epigenetic and maternal-placental factors, leading to possible multiple organ damage. Because of this high susceptibility to OS, the term Free Radicals Disease (FRD) was coined for a series of typical prematurity-related diseases in which oxidative damage plays a determining role, demonstrable through the dosage of OS biomarkers. Elevated levels of TH, AOPP and NPBI in cord blood have been found to correlate with an increased risk for FRD [69.] (Fig. 3).

High TH plasma levels were found in *preterm hypoxic newborns* providing indirect evidence of an increase in FRs generation during hypoxic conditions. The correlation between TH and hypoxanthine in plasma of preterm newborn strongly suggests that the deeper the hypoxia, the greater is the reactive oxygen metabolite production. The degree of hypoxia correlated with AOPP levels, indicating that plasma proteins are impacted in FRs damage in preterm hypoxic newborns [35.], [36.].

Even in the most frequent complication of preterm, the *respiratory distress syndrome (RDS)* the role of OS has been demonstrated: MDA, protein carbonyls and 7,8-OHdG showed a significant increase with depleted levels of total antioxidant capacity (TAC) in neonatal RDS when compared to healthy newborns [70.]. In a recent study, significant higher plasma levels of protein carbonyls have been confirmed in preterm neonates with RDS compared to healthy preterm newborns [71.].

Over the last decades, emerging data have suggested that OS is involved in the lung development and in the development of *bronchopulmonary dysplasia (BPD)* in the preterm newborn, and that the lung injury process leading to BPD occurs within hours to days from delivery with the oxidation representing a major contributor to the process. Neonatal lung injury has multiple etiologic factors that act in a synergic way; a complex interrelation between inflammation and OS represents the physiopathological background predisposing an immature lung to BPD development, with an important contribute due to the premature exposure to hyperoxia [72.], [73.].

Human studies have shown a quantitative increase in oxidative damage to pulmonary proteins and lipids and decrease in levels of antioxidants in biological fluids of ventilated preterm infants [74.]. In particular, preterm infants who later developed BPD and those mechanically ventilated with high oxygen requirement have shown higher carbonyl content in pulmonary fluid than those who did not developed BPD or requiring less oxygen [75.], [76.]. Collard et al. demonstrated in the epithelial lining fluid of preterm infants who developed BPD significantly higher concentration of MDA than those who were not oxygen dependent [77.]. More recently, urinary levels of 7,8- OHdG in preterm babies who later developed BPD were found higher compared with those of infants who did not developed BPD, with a positive correlation between these levels on the third day of life and the duration of mechanical ventilation [78.].

Hyperoxia and oxidative stress are also called into question in the physiopathology of *retinopathy of prematurity (ROP)*, a major cause of visual impairment in preterm infants. The role of OS in this condition is complex and interrelated to inflammatory, angiogenetic, metabolic, and genetic factors. In a previous study, the Authors did not found significant difference in markers of OS (NPBI, TH, AOPP and carbonyl groups) between preterm babies with ROP stage 1–2 and babies without ROP. However, a significant decrease in the NPBI levels and increased TH in the first 3 weeks in both groups was found, suggesting that all preterm infants are physiologically prone to OS at birth because the extrauterine environment is richer in oxygen than intrauterine one [79.]. Moreover, other Authors have found a significant difference in leukocyte and urine 7,8-OHdG levels and in plasma and urine MDA levels in patients with ROP compared to those without ROP, making them useable as possible screening tools for ROP [80.].

Several studies have suggested a role of OS in the pathogenesis of *Necrotizing Enterocolitis (NEC)* [81.], [82.]. Aydemir et al. found that preterm infants with NEC had significantly higher total oxidant status (TOS), and oxidative stress index (OSI) levels compared with controls without NEC, with higher levels of TOS and OSI being associated with the severity of NEC [83.]. Furthermore, a strong association between the concentration of OS markers in cord blood and the occurrence of NEC in preterm infants was found; in particular, AOPP, TH and NPBI cord blood levels were significantly higher in babies with NEC than in

Table 1
OS biomarkers in fetal and neonatal diseases.

Biomarkers	*Fetal-placental or pregnancy disease*	*Neonatal disease*	*Biological fluid/tissue*
NPBI	Chorioamnionitis (CA)/placental vascular underperfusion (VU)	ROP NEC IVH-PHVD/Brain injury HIE in term babies	Cord blood and/or newborn's peripheral vein [64,68,78,83,91]
TH		Hypoxia in preterm ROP NEC Kidney damage	Cord blood and/or newborn's peripheral vein [33,35,68,78,83,85]
AOPP	Chorioamnionitis (CA)/placental vascular underperfusion (VU)	Hypoxia in preterm ROP NEC HIE in term babies	Cord blood and/or newborn's peripheral vein [35,36,65,69,79,84,86,92]
Carbonyl groups	GDM	ROP RDS BPD	Placenta [60] Pulmonary fluid [75,76] Cord blood [70] Newborn blood [71]
F2-IsoPs	FGR/IUGR Preeclampsia GDM Maternal overweight/obesity pPROM Down Syndrome Chorioamnionitis (CA)/placental vascular underperfusion (VU)	HIE in term babies PDA SGA Respiratory morbidity and neurodevelopmental outcome	Amniotic fluid [43,66–68] Placenta [52,57] Maternal plasma [53,67] Cord blood [61,65] Newborn's peripheral vein and/or newborn urine samples [88,89,91,92]
7,8-OHdG	Preeclampsia + FGR/IUGR	ROP RDS BPD	Placental trophoblast cells [54,55] Cord blood [70] Newborn blood and urine samples [78,80]
MDA	FGR/IUGR GDM	ROP RDS BPD	Maternal plasma, cord blood, placental tissue [46,47,54,70] Newborn blood and urine samples [80] Pulmonary fluid [77]

babies without [84.]. Ozdemir et al. reported also a significant increase of intestinal MDA in an animal model (neonatal rats) of NEC [85.]. NEC is a multifactorial disease with a poorly understood pathogenesis [81], of which however oxidative stress and inflammation are closely related key-elements. Many inflammatory biomarkers (cytokines i.e. IL-6, IL-8/CXCL8, IL-10, and IL-18) have been studied, however the diagnostic accuracy is not optimal and nowadays none single biomarker has been identified as a stand-alone diagnostic test with rapid turnaround suitable for clinical practice [86.].

OS has been found to play a key role also in post-ischemic *kidney damage*. In the first two weeks of life, AOPP and TH significantly correlated with alpha-1 microglobulin and N-acetyl-b-D-Glucosaminidase (a microprotein and a tubular enzyme, respectively, that represent a clinical tool for assessing tubular dysfunction), as expression of oxidative stress-induced kidney damage in preterm infants [87.].

Patent Ductus Arteriosus (PDA) is a possible complication during the course of prematurity, which can be related in turn to other conditions like kidney damage, NEC, BPD or intraventricular hemorrhage. Urinary isoprostane levels have shown significant changes in preterm with PDA from before treatment with ibuprofen to after treatment. We found a significant decrease in IsoPs 12–24 h after pharmacological closure with ibuprofen, followed by a rebound on the seventh day after treatment, suggesting the potential antioxidant effect of the studied drug in preterm babies with PDA who are at high risk for OS [88.]. Inayat et al. have partially confirmed these results in a recent study, finding increased isoprostane (8-isoPGF2α) levels post-treatment probably due to increased oxygenation and ROS levels, although they report lower pretreatment IsoPs levels in preterm infants who developed hemodynamically significant PDA [89.].

The probably most studied FRD of prematurity is the *intraventricular hemorrhage (IVH)*, a common disease associated with long-term consequences together with the possibly subsequent *periventricular leukomalacia (PVL)*. Because it is now accepted that FRs persists to the damage of the premature brain, to prevent long-term sequelae of OS, an early diagnosis of the presence of an OS-damage by a validate panel of biomarkers is necessary [90.]. A significant association between TH, AOPP and NPBI levels in cord blood of preterm and the development of all grades of IVH has been demonstrated; this finding has suggested that increased OS markers are a direct index of increased production of FRs in central nervous system as a response to oxidative neuronal damage [69.]. NPBI has been found increased in cerebrospinal fluid of preterm infants with post-hemorrhagic ventricular dilatation- PHVD, suggesting an association between IVH and subsequent white matter damage [16.]. Moreover, NPBI seems to be the best early predictive marker of neurodevelopmental outcome [14.]. Isoprostanoids are other reliable biomarkers of brain injury [9.]. An interesting recent study has shown that in the first month after birth, increases in plasma IsoPs identify preterm infants at risk for respiratory morbidity at term equivalent age and for worse developmental outcomes at 12 months of corrected age, with poor neurodevelopment largely independent of respiratory morbidity [91.].

Not only the preterm but also the term brain is particularly vulnerable to the OS-related damage. The predictive role of a default panel of OS biomarkers for the early identification of infants at high risk of *hypoxic-ischemic encephalopathy (HIE)* and their validation through the correlation with MRI findings was recently reported. 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. This finding 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 h, it suggests 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 [92.]. In a recent study, NPBI also showed to be associated with non-parenchymal brain injury in neonates after surgery [93.].

2. Conclusions

Oxidative stress can occur early in pregnancy, and continue during the prenatal and the postnatal period. It is involved in the pathogenesis of several diseases of the newborns, especially in the preterm ones. Numerous biomarkers have been investigated in the fetus and in the newborns (Table 1).

In the clinical practice, the routine use of reliable markers of OS could be useful in the early identification of children at highest risk of tissue damage, in devising strategies to prevent or ameliorate perinatal outcome and in predicting the potential efficacy of therapeutic strategies aimed at reducing such an OS. However, when examining predictors in infants suffering OS it is important to assess whether old predictors are valid with new thresholds. Furthermore, it is important to keep in mind that oxidative stress is a nuanced phenomenon, difficult to characterize, and one biomarker is not necessarily better than others. The vast diversity in OS between diseases and conditions has to be taken into account when selecting the most appropriate biomarker [94].

In summary, few of the biomarkers reported to date have been qualified, but the development of additional biomarkers is warranted. New proteomic and metabolomics technologies and their correlation with OS biomarkers could be an interesting object of research and merit further investigation.

Authorship

Serafina Perrone contributed to conception and design of the study, to revise the manuscript critically. Elisa Laschi contributed to drafting the review. Giuseppe Buonocore contributed to revising the manuscript critically and to final approval of the version to be published.

Conflicts of interest

The Authors state that no conflict of interest exists regarding the described work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2019.03.034.

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ANNEX 9

Perrone S., Laschi E., Buonocore G. *Oxidative stress biomarkers in the perinatal period: diagnostic and prognostic value.* Semin Fetal Neonatal Med. 2020 Apr; 25(2):101087.
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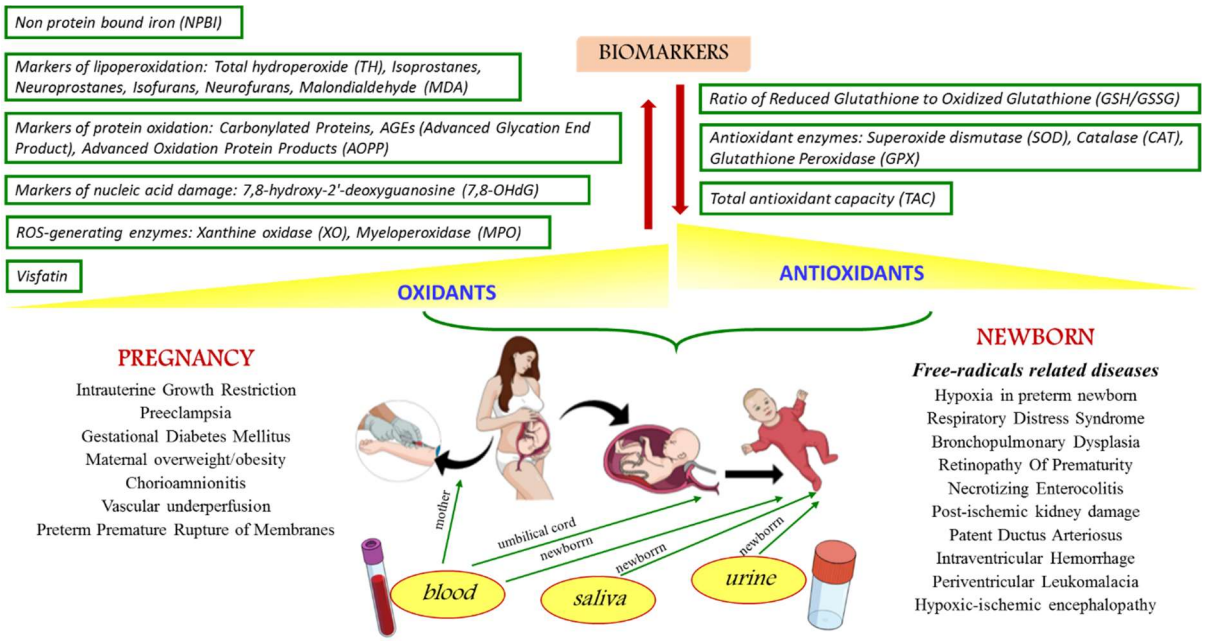
ABSTRACT

Perinatal oxidative stress (OS) is involved in the physiopathology of many pregnancy-related disorders and is largely responsible for cellular, tissue and organ damage that occur in the perinatal period especially in preterm infants, leading to the so-called "free-radicals related diseases of the newborn". Reliable biomarkers of lipid, protein, DNA oxidation and antioxidant power in the perinatal period have been demonstrated to show specificity for the disease, to have prognostic power or to correlate with disease activity. Yet potential clinical applications of oxidative stress biomarkers in neonatology are still under study. Overcoming the technical and economic difficulties that preclude the use of OS biomarkers in the clinical practice is a challenge that needs to be overcome to identify high-risk subjects and to predict their short- and long-term outcome. Cord blood, urine and saliva represent valid and ethically acceptable biological samples for investigations in the perinatal period.



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1. Perinatal oxidative stress: the subtle balance between physiology and disease

Perinatal oxidative stress (OS) is the inevitable condition of life in an oxygen-rich atmosphere and reactive oxygen species (ROS) generated under certain conditions especially in the mitochondria of all cell types are the main triggers of OS. ROS and other free radicals (FRs), highly reactive chemical species that can rapidly interact with intracellular biological molecules, are indispensable for the cellular physiological functions under normal conditions, but a fragile redox balance exists. According to Lushchak, "oxidative stress is a situation when steady-state ROS concentration is transiently or chronically enhanced, disturbing cellular metabolism and its regulation and damaging cellular constituents" [1], so OS occurs when the FRs production is not adequately balanced by the intracellular antioxidant systems. The dangerous effects of FRs are due to their property of being very unstable molecules and their ability to react with polyunsaturated fatty acids of cell membranes, proteins, polysaccharides and nucleic acids [2]; any situation where a lack of antioxidant systems exists can lead to functional alterations within the cells.

The perinatal period, which commences at 22 completed weeks (154 days) of gestation and ends seven days after birth according to the World Health Organization (WHO) definition [3], represents a critical period for the subtle redox balance. Foetuses and newborns are highly subjected to oxidative damage, due to their aerobic metabolism linked to the rapidly growing energy demand, the presence of conditions leading to excessive FRs production (hypoxia and asphyxia, ischemia-reperfusion, hyperoxia, inflammation, mitochondrial impairment, increased free circulating non protein-bound iron, drugs), and the immaturity of antioxidant systems [4].

For this reason, OS is involved in the physiopathology of many pregnancy-related disorders and is largely liable for cellular, tissue and organ damage that occurs in the perinatal period especially in preterm infants, leading to the so-called "free-radicals diseases" (FRD) like retinopathy of prematurity (ROP), bronchopulmonary dysplasia (BPD), intraventricular haemorrhage (IVH), periventricular leukomalacia (PVL), necrotizing enterocolitis (NEC), kidney damage [2,5] but also respiratory distress syndrome (RDS), patent ductus arteriosus (PDA), and hypoxic-ischemic encephalopathy (HIE).

The relevant interest in OS in the perinatal period does not only concern short-term complications, but is also justified by the fact that OS is strongly involved in fetal programming of adult diseases, through the gene expression regulation and cell growth modulation. OS may be the general underlying mechanism that links altered placental function to fetal programming, and it can also be hypothesized that this programming process is extended into early postnatal life for premature infants [6].

In this perspective, the identification of reliable biomarkers in the

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perinatal period appears to be essential for the early discovery of FRD and the subsequent long-term health outcome.

2. Oxidative stress biomarkers: what are they?

2.1. Definition, properties and types of OS biomarkers

According to the WHO, "a biomarker is any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease"; it should be relevant and valid [7]. Therefore, a clinically useful biomarker should be able to be measured in a reliable and reproducible way (it must be reasonably stable and present in accessible tissue or fluid), but it should also satisfy at least one requirement such as showing specificity for the disease, having prognostic power or correlating with disease activity [8]. One of the weaknesses of current science is the lack of validated and clinically applicable OS biomarkers; the need for reliable, validated and low-cost biomarkers has received increasing attention over the last few decades, yet their potential clinical applications are still unclear especially in perinatology.

FRs reactions normally occurring in living organisms lead to the production of different FRs species: oxygen-centred radicals (ROS) to a greater extent, but also nitrogen-centred radicals (reactive nitrogen species, RNS) and sulphur-centred radicals. OS is difficult to quantify in vivo because ROS, RNS and other FRs have a very short half-life. Analytical methods available to study OS include those aimed at detecting potential risk of oxidative stress, such as non protein bound iron (NPBI) with its capacity to generate $^{\circ}OH$ through Fenton reaction, and those aimed at detecting direct oxidative damage to lipids, proteins and DNA (Fig. 1). In addition, stress response proteins, ROS-forming enzymes like xanthine oxidase (XO), uncoupled nitric oxide synthases (NOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), and the activity of antioxidant enzymes like superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) can be used as OS biomarkers.

2.2. Known, potentially available and reliable OS biomarkers

2.2.1. Non protein-bound iron (NPBI)

Iron can catalyse the formation of the highly reactive $^{\circ}OH$ from H_2O_2 in the Fenton reaction: $Fe^{2+} + H_2O_2 => Fe^{3+} + {}^{\circ}OH + OH^{-}$. When bound to or stored in proteins, it is an essential element for aerobic metabolic processes but it is toxic when unbound. The term non protein-bound iron indicates a low molecular mass iron form, free from the high-affinity binding to plasma proteins like transferrin and thus available to react with reduced intermediates of O_2 [9]. Reliable measurements of NPBI are possible in studies on oxidative stress under experimental and clinical conditions, through a method based on preferential chelation of NPBI by a large excess of the low-affinity ligand nitrilotriacetic acid [10].

2.2.2. Markers of lipid peroxidation

Cellular membranes containing a high proportion of polyunsaturated lipids are susceptible to FRs-attack and oxidation. Polyunsaturated fatty acid (PUFA) contained in phospholipids undergo a reaction with O_2, producing lipid hydroperoxides. *Total hydroperoxide (TH)* represents a measure of overall OS: lipids and proteins' FRs-exposure leads to hydroperoxides generation, and these can produce secondary reactive radical species that can be measured, but undergo rapid degradation in vitro. A series of prostaglandin-like compounds called isoprostanes (IsoPs) and neuroprostanes (NPs) represent more stable lipid peroxidation markers; they originate from oxidation of arachidonic acid (AA) and docosahexaenoic acid (DHA), respectively. *F2-isoprostanes* result from in vivo and in vitro peroxidation of AA and phospholipids by FRs reactions; an oxygen insertion step diverts intermediates from the IsoPs pathway to form other compounds, termed *isofurans (IsoFs)* that contain a substituted tetrahydrofuran ring; since these are also chemically stable, they can act as in vivo biomarkers of oxidative damage [4,5]. Because of this differential method of formation, oxygen tension can affect lipid peroxidation profile, and so the ratio IsoFs/IsoPs provides information about the relative oxygen tension where the lipid peroxidation is occurring [11]. DHA, a major component of neuronal membranes, oxidizes both in vitro and in vivo to form F2-IsoP-like compounds termed *F4-neuroprostanes* (*F4-NPs*), in vivo-biomarkers of oxidative damage selective for neurons.; similarly

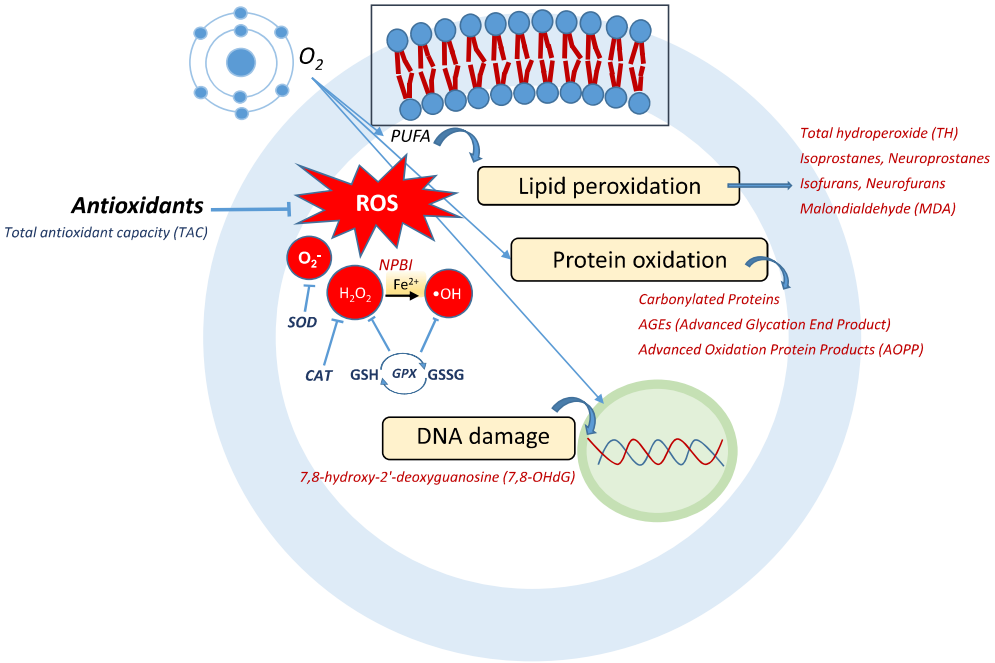

Fig. 1. Oxidative stress and oxidative stress biomarkers in the cell.

to IsoFs, an alternative pathway of oxidation of DHA leading to the formation of IsoFs-like compounds termed *neurofurans* (*NFs*) [4,5]. The currently most often used method to quantify F2-IsoPs and IsoFs in biological tissues and fluids is gas chromatography/mass spectrometry (GC/MS); a variety of analytical procedures to measure F2-IsoPs, including other GC/MS methods and liquid chromatography/MS and immunological approaches, are reported in the literature [12]. Adapting the methodology used to quantitate the F2-IsoPs, Arneson and Roberts have described methods to quantify both the F4-NPs and the NFs, using stable isotope dilution, negative ion chemical ionization GC/MS [13].

Malondialdehyde (*MDA*) is one of the most studied low-molecular-weight end-products of polyunsaturated fatty acid peroxidation, highly cytotoxic because of its ability to bind proteins or nucleic acids very quickly [14]; it is a constituent of the so called thiobarbituric acid reactive substances (TBARS). The TBAR test, a reaction of thiobarbituric acid with MDA and other carbonyl compound, has been frequently used to assess MDA concentrations even if it lacks specificity; recently, the use of ultra-high-performance liquid chromatography-high-resolution MS (UHPLC-HRMS) for the quantification of free and total plasmatic MDA using dinitrophenylhydrazine (DNPH) as the derivatizing agent has been validated [15].

2.2.3. Markers of protein oxidation

A large number of different proteins and amino-acidic residues represent a target for ROS and RNS. FRs can modify aminoacidic residues of proteins and lead to cross-linking, changes in conformation and loss of function. Oxidative modifications include oxidation of sulphur-containing residues, hydroxylation of aromatic and aliphatic groups, nitration of tyrosine residues, nitrosylation and glutathionylation of cysteine residues, chlorination of aromatic groups and primary amino groups, and conversion of some amino acid residues to carbonyl derivatives [16]. Oxidative damaged proteins, if not removed rapidly by proteases, can accumulate to readily detectable levels; several methods have been developed for the detection of such modified proteins [16–18]. During the oxidation of proteins, carbonyl groups (-CO═O) are introduced into the side-chains of the proteins. The measure of carbonyl levels in proteins is the most widely used marker of oxidative protein damage, and tissues injured by oxidative stress generally contain increased concentrations of *carbonylated proteins*. Lysine, histidine, and cysteine residues are involved in this “carbonyl” stress, because they can react with lipid peroxidation products like MDA leading to the formation of carbonyl derivates called *AGEs* (Advanced Glycation End Product), a group of heterogeneous molecules such as pentosidine [4,16]. The concentration of carbonyl groups is a good measure of OS because of their relatively early formation and their stability. Many assays are available to detect protein carbonyls; highly sensitive methods involve derivatization of the carbonyl groups with DNPH, which leads to formation of a stable product easily detectable with spectrophotometric assay, enzyme-linked immunosorbent assay-ELISA, or one-dimensional/two-dimensional electrophoresis followed by Western blot immunoassay [17]. AGEs’ assays are mostly based on the use of specific antibodies or spectrofluorometric measurements based on their fluorescent properties [16].

Advanced oxidation protein products (AOPP) are the terminal products of protein exposure to FRs without oxidant properties and they represent a marker of the degree of protein damage in OS; AOPP include protein aggregates by disulphide bridges and/or tyrosine cross-linking. They can be measured using spectrophotometry on a microplate reader [18] or by colorimetric tests using a chloramine standard or human serum albumin derivative [16].

2.2.4. Biomarkers of DNA oxidation

7,8-hydroxy-2′-deoxyguanosine (7,8-OHdG) is a reliable and frequently used marker of OS-related DNA damage, a guanosine base oxidation product. It can be detected in human tissues or samples and in human peripheral leukocytes, by an ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method [19,20].

2.2.5. Ratio of reduced glutathione to oxidized glutathione (GSH/GSSG)

Glutathione (GSH) is a tripeptide representing the most abundant intracellular nonprotein thiol, acting as an antioxidant system by its ability to scavenge ROS through the reversible oxidation to its disulphide GSSG, which in turn can be reduced to GSH by the activity of glutathione reductase (GR) and the reducing power of NADPH. GSH levels and the ratio GSH/GSSG, normally ranging from 30 to 100, decrease in case of OS. The measurement of GSH, GSSG, and their ratio has been considered an index of the redox status and so as a useful marker of diseases in humans [16]. They can be measured in biological fluids through different methods like spectrophotometry, HPLC, capillary electrophoresis, nuclear magnetic resonance, and MS [21], even if known methodological artefacts reduce its power. Recently, a Spanish group developed reliable methods to obtain accurate and reproducible determinations [22,23].

2.2.6. ROS-generating enzymes: XO, MPO

Some ROS-generating enzymes are normally present in cells and involved in several functions, but can also be responsible for an oxidative burden according to the levels of their substrates and cellular antioxidant defences. *Xanthine oxidase (XO)* catalyses the oxidation of xanthine to uric acid and is a well-known source of superoxide O2-. The enzyme exists in the two forms, an oxidase XO (that oxidizes xanthine to uric acid using O2 and producing H2O2) and a dehydrogenase XDH (that carries out the same reaction using NAD+). Under hypoxic conditions, XDH is rapidly released in circulation and converted to XO, with subsequent amplification of ROS production [8,16]. *Myeloperoxidase (MPO)* is a heme peroxidase that catalyses the reaction between H2O2 and chloride ions with the production of ROS, producing hypochlorous acid (HOCl) as primary oxidant. Oxidant species derived from MPO lead to generate specific oxidation products, such as 3-chlorotyrosine (3-Cl-Tyr), an AOPP that can be used as biomarker. A limitation of using MPO as a biomarker is that current methods are not standardized between laboratories and do not provide direct information on MPO activity [8,16].

2.2.7. Antioxidant enzymes: SOD, CAT, GPX

Superoxide dismutase (SOD) is the first detoxification enzyme and most powerful antioxidant in the cell, acting as a component of the first line defence system against ROS. It catalyses the dismutation of O− superoxide anion to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), rendering the potentially harmful superoxide anion less hazardous [24]. There are different isoforms of SOD: copper-zinc superoxide dismutase (SOD1), with copper and zinc in its catalytic core which is localized in the intracellular cytoplasmic compartments, and manganese superoxide dismutase (SOD2) which plays an important role as a primary mitochondria antioxidant enzyme. SOD activity can be measured by analyzing the inhibition in the rate of reduction of a tetrazolium salt by O^- generated through a xanthine/XO enzymatic system [25]. SOD acts also as a pro-oxidant producing H_2O_2; therefore, other antioxidant enzymes such as CAT and GPX are required and an imbalance in their ratio may be dangerous [16]. *Catalase (CAT)* is a common antioxidant enzyme, located primarily in the peroxisomes but absent in mitochondria, that catalyses the conversion of H_2O_2 into H_2O and O_2; its enzymatic activity can be measured by several colorimetric/spectrophotometric assays [25]. *Glutathione Peroxidase (GPx)* is an important intracellular enzyme that breakdown H_2O_2 to water and lipid peroxides to their corresponding alcohols mainly in the mitochondria, through the oxidation of GSH to GSSG; GPX activity can be measured using cumene hydroperoxide and GSH as substrates [16,24].

2.2.8. Total antioxidant capacity (TAC)

The non-enzymatic antioxidant capacity or *total antioxidant capacity*

(TAC) is defined as the moles of oxidants neutralized by 1 L of body fluids. In plasma, non-enzymatic antioxidants include endogenous and nutritional compounds, like bilirubin and thiols on one side and tocopherols, ascorbic acid, carotenoids, and phenolics on the other [16]. Various analytical methods have been developed to measure TAC, including the 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical scavenging assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, ferric reducing antioxidant power (FRAP) assay, and oxygen radical absorbance capacity (ORAC) assay. The principle of the conventional TAC assay methods, used for their easy and fast characteristics, is based on the radical scavenging activity and redox potential of antioxidants. The ABTS assay seems to be an appropriate method to measure overall plasma antioxidant capacity and predict the body's antioxidant status in humans [26].

2.2.9. Visfatin

Visfatin, also known as nicotinamide phosphoribosyl transferase (NAMPT), is a ubiquitous adipokine secreted from visceral fat, or better a multi-functional molecule that can act intracellularly and extracellularly as an adipokine, cytokine and enzyme. In recent years, visfatin has been described as a potent marker of inflammation and dysfunction and has been associated with OS [27], even if its pathophysiological role in humans remains largely unknown. Visfatin can be assessed with enzyme-linked immunosorbent assay (ELISA) and has recently been investigated as a new biomarker of OS in preterm neonates [28].

3. OS biomarkers in perinatology: a long history between research and clinical practice

3.1. OS in the placenta, in the foetus and newborn: clinical correlations

The peculiar vulnerability of the foetus and newborn to oxidative insults has increasingly encouraged the search for new biomarkers for the perinatal period that can help to identify babies at increased risk and prevent short- and long-term effects.

Since pregnancy, the fragile redox balance has an important role in both appropriate growth and development of the placental-fetal unit and in the pathogenesis of pregnancy-related diseases [29]. An increase of various OS biomarkers has been observed in several complications of pregnancy: intrauterine growth retardation (IUGR) or fetal growth restriction (FGR) [30–33], preeclampsia (PE) [33–36], gestational diabetes mellitus (GDM) [37–40], maternal overweight or obesity [41,42], chorioamnionitis (CA) or vascular underperfusion (VU) often associated with preterm labour [43], and preterm premature rupture of membranes (pPROM) [44,45]. It is known that all these conditions can already affect the subjective health and the long-term outcome, according to the fetal programming theory [6].

In addition to this predisposing background, another crucial event in everyone's life is the moment of foetal-to-neonatal transition. Initiation of breathing immediately after birth triggers profound cardiorespiratory and metabolic changes and the foetal-to-neonatal transition increases exponentially the availability of oxygen to tissues causing a physiological oxidative stress. In pathological conditions such as hyperoxia, hypoxia-reoxygenation and hypoxia, a severe oxidative stress occurs and leads to pathological conditions [46,47]. Oxidative damage can also extend and amplify with the persistence of pro-oxidizing factors beyond the moment of birth, during the neonatal period. For this reason, an increase of OS biomarkers has been observed also in several neonatal conditions, both in preterm and term neonates: preterm hypoxic newborns [48], respiratory distress syndrome (RDS) [49,50], bronchopulmonary dysplasia (BPD) [51–55], retinopathy of prematurity (ROP) [56,57], necrotizing enterocolitis (NEC) [58–60], post-ischemic kidney damage [61], patent ductus arteriosus (PDA) [62,63], intraventricular haemorrhage (IVH) and periventricular leukomalacia (PVL) [64–66], perinatal asphyxia and hypoxic-ischemic encephalopathy (HIE) [67,68]. The role of OS in the genesis of brain damage is of particular and ever-increasing interest [69], and a correlation between brain injury and neuroimaging has also been demonstrated [68].

Moreover, the effects of the oxidative damage in utero, at birth and in the newborn have repercussion beyond the perinatal period. This is evidenced, for example, by a recent study showing that in the first month after birth, increases in OS biomarkers identify preterm infants at risk of respiratory morbidity at term equivalent age and for worse developmental outcomes at 12 months corrected age [70]. Similarly, some authors have demonstrated a significant correlation between an increase in OS biomarkers (8-OHdG and 8-isoPGF) and neurodevelopmental outcome at 18 months corrected age in VLBW infants [71].

3.2. Non-invasiveness and choice of biological samples: an ethical problem in perinatology

The correlations between OS and short- and long-term diseases demonstrated in clinical and experimental studies justify the increasing need for developing reliable tools to evaluate, control and reduce OS in newborns, especially preterms. The OS biomarkers known to date have been studied and measured in several different biological fluids and tissues, most of which were blood samples (plasma or serum). In the perinatal field, a peculiar and relevant aspect to consider is the need for non-invasiveness and the possibility to measure biomarkers reliably in small quantities of biological samples. Recently, some authors reviewed OS biomarkers determined in non-invasive samples as oxidative status evaluation in preterm infants, considering biological fluids like cord blood, urine and saliva [72].

Cord blood and placenta represent important sources of OS biomarkers that can consent to identify early high-risk newborns. Many foetal-neonatal OS biomarkers and their correlation with pregnancy related disease or FRD have been studied in these tissues [31, 35–41, 43,48–49, 59–60,66], and recently reference values for isoprostanes, AOPP and NBPI in cord blood have been established [73]. Their only disadvantage is not to consent follow-up studies along some because new samples are needed in order to monitor changes along time [72]. For this reason, urine and saliva samples of newborns can represent valid and ethically acceptable alternatives. Reference values for isoprostanes in urine of preterm infants not undergoing any severe conditions have been published [74].

3.3. Applicability of OS biomarkers in perinatal clinical practice: which reality?

Another important aspect relative to OS biomarkers in perinatology concerns the methods used to determine them. Most of the analytical methods applied to determine OS biomarkers in non-invasive samples are based on immunoassays, colorimetric assays, commercial kits and spectrophotometric measures, and these techniques have low sensitivity and specificity, high number of interferences and low precision. Some of the methods used for research in preterm infants are based on mass spectrometry, and these are not easily suitable for clinical practice because they are expensive and require specialized personnel. Finally, other studies used methods like ELISA or spectrophotometry that are easily applicable to clinical practice but still need to be validated clinically, and their pathological values should be well established for different diseases as potential biomarkers [72]. So, the complex methodology, the elevated cost and the difficulty in interpreting data from a clinical point of view has limited until now the use of OS biomarkers to experimental and clinical research, precluding their use in clinical routine [47].

Nowadays, none of the biomarkers studied and listed above is actually used in clinical practice but the growing research on this topic will allow collecting as much data as possible from both a technical and a clinical interpretation point of view.

4. Conclusions

The OS has a crucial physio-pathological role during pregnancy, foetal-to-neonatal transition, and the neonatal period. The relevance of OS biomarkers in the foetus and newborn is well established, in order to identify high-risk subject and to predict their short- and long-term outcome. Overcoming the technical and economic difficulties that preclude the use of OS biomarkers in the clinical practice is a challenge that needs to be overcome in a short time, because an accurate evaluation of OS and the possibility to implement effective treatments would contribute to improve the quality of care of neonatal patients.

- Perinatal oxidative stress (OS) is involved in the physiopathology of many perinatal disorders
- Biomarkers of lipid, protein, DNA oxidation and antioxidant power have been demonstrated to show specificity for the disease, to have prognostic power or to correlate with disease activity
- Technical and economic difficulties still preclude the use of OS biomarkers in clinical practice
- Cord blood, neonatal urine and saliva represent valid and ethically acceptable biological samples for oxidative stress investigations in perinatal period.

Declaration of competing interest

The Authors state that no conflict of interest exists regarding the described work.

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ANNEX 10:

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Research Article

Antioxidant Effect of Melatonin in Preterm Newborns

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Introduction. Preterm infants are at risk of free radical-mediated diseases from oxidative stress (OS) injury. Increased free radical generation has been demonstrated in preterm infants during the first seven days of life. Melatonin (MEL) is a powerful antioxidant and scavenger of free radicals. In preterm neonates, melatonin deficiency has been reported. Exogenous melatonin administration appears a promising strategy in the treatment of neonatal morbidities in which OS has a leading role. *Objective*. The aim was to evaluate plasma MEL concentrations and OS biomarkers in preterm newborns after early administration of melatonin. *Methods*. A prospective, randomized double-blind placebo-controlled pilot study was conducted from January 2019 to September 2020. Thirty-six preterm newborns were enrolled. Starting from the first day of life, 21 received a single dose of oral melatonin 0.5 mg/kg once a day, in the morning (MEL group); 15 newborns received an equivalent dose of placebo (placebo group). Samples of 0.2 mL of plasma were collected at 24 and 48 hours after MEL administration. Plasma concentrations of melatonin, non-protein-bound iron (NPBI), advanced oxidation protein products (AOPP), and F2-isoprostanes (F2-Isopr) were measured. Babies were clinically followed until discharge. *Results*. At 24 and 48 hours after MEL administration, the MEL concentrations were significantly higher in the MEL group than in the placebo group (52759.30 ± 63529.09 vs. 28.57 ± 46.24 pg/mL and 279397.6 ± 516344.2 vs. 38.50 ± 44.01 pg/mL, respectively). NPBI and AOPP did not show any statistically significant differences between the groups both at 24 and 48 hours. At 48 hours, the mean blood concentrations of F2-Isopr were significantly lower in the MEL group than in the placebo group (36.48 ± 33.85 pg/mL *vs.* 89.97 ± 52.01 pg/mL). *Conclusions*. Early melatonin administration in preterm newborns reduces lipid peroxidation in the first days of life showing a potential role to protect high-risk newborns. *Trial Registration*. This trial is registered with NCT04785183, Early Supplementation of Melatonin in Preterm Newborns: the Effects on Oxidative Stress.

1. Introduction

Preterm infants are at risk for neonatal disorders related to immaturity. A common factor in the pathogenesis of such diseases is the free radical-mediated tissue injury derived from oxidative stress (OS) [1]. The endogenous indoleamine melatonin, synthesized from the neurotransmitter serotonin, is a powerful broad-spectrum antioxidant and readily available scavenger of free radicals. Foetal melatonin has a maternal origin, and after birth, the full-term neonates have an irregular melatonin secretion for 3-5 months, leading to a transient melatonin deficiency in the neonatal period and in the first months of life. Prematurity delays the maturation of the neurological network that controls melatonin secretion, leading to poor secretion for an even longer period. Furthermore, the onset of pineal melatonin secretion seems to be even more delayed in case of neurological damage, and this event, together with other predisposing conditions, makes the preterm even more susceptible to the free radical-mediated damage [2–4]. Therefore, exogenous melatonin administration appears a promising strategy in the treatment of neonatal morbidities in which OS has a leading role. Moreover, as it shows neuroprotective properties, it was present as a joint therapy in addition to hypothermia after hypoxic-ischemic

encephalopathy [5–8]. Several studies tested the efficacy of melatonin to counteract oxidative damage in diseases of newborns such as chronic lung disease, perinatal brain injury, necrotizing enterocolitis, retinopathy of prematurity, and sepsis, giving promising results [9–11]. In these studies, the dosages of melatonin varied over a wide range, ranging from 0.1 to 100 mg/kg. This is an evidence that the pharmacokinetic profile of melatonin is better known in adults than newborns [12]. Indeed, just few studies investigated pharmacokinetic characteristics of melatonin in preterm and asphyxiated neonates. Merchant et al. observed and described a decreased volume of distribution and prolonged half-life and clearance of the melatonin in preterm infants with respect to adults and older children. Melatonin was administered intravenously at the dosage of 0.1 mg/kg for two hours [13]. Carloni et al. investigated the melatonin pharmacokinetics at comparable doses after intragastric administration in human preterm infants. The main result of the study was that a single intragastric bolus of 0.5 mg/kg of melatonin resulted in higher serum melatonin level than adults suggesting the possibility to get and keep therapeutic concentrations with this dose [14]. Finally, Balduini et al. demonstrated that a safe and potentially effective dose of melatonin for infants with hypoxic ischemic encephalopathy undergoing hypothermia should not exceed 5 mg/kg, depending on the route of administration [15]. However, no data are available on the therapeutic efficacy of these specific doses. The aim of this study was to evaluate melatonin concentrations and biomarkers of oxidative stress in preterm infants after early administration of melatonin.

2. Materials and Methods

This prospective randomized double-blind placebo-controlled pilot study was conducted at the Neonatology Unit of the Polyclinic in Messina from January 2019 to September 2020. The study was approved by local Ethical Committee (protocol number 42/2018). Written informed consent was obtained from parents. Inclusion criteria were gestational age < 37 weeks and normal liver and kidney function tests. Exclusion criteria are all outborn babies, babies with severe congenital malformations, sepsis, inborn errors of metabolism, suffering from perinatal hypoxia, or born from mothers with mental disorders, to eliminate conditions that could affect melatonin production. Additional exclusion criteria were as follows: withdraw informed consent, insufficient blood sample, and hemolysis of the blood sample. The MEL group received an oral dose of 0.5 mg/kg of melatonin, once a day in the morning, in the first week of life; the placebo group received 0.5 mL of 5% glucose solution. Newborns received melatonin (Pisolino® Gocce, Pediatrica, Italy) by a nasogastric tube. Pisolino® Gocce contains fructose, purified water, potassium sorbate, sodium benzoate, flavorings, and xanthan gum. The product is present in the register of food supplements of the Ministry of Health website (http://www.ministerosalute.it/alimenti/dietetica) and classified with the following code: 62853.

This product is subject to the European Directive on foods according to the DL n. 169 of 21/05/2004 and not to the European Directive on medicines 2001/20/EC implemented at Italian level with D.L. n. 211 of 06/24/2003. Melatonin administration has a good safety profile, with no known adverse effects [16]. Plasma concentrations of non-protein-bound iron (NPBI) (micromol/L), advanced oxidation protein products (AOPP) (micromol/dL), and F2-isoprostanes (F2-Isopr) (pg/mL) were determined at 24 and at 48 hours after administration of melatonin. The primary endpoint was to evaluate MEL concentration in the MEL group and placebo group. The secondary endpoint was to evaluate biomarkers of OS, such as AOPP, NPBI, and F2-Isopr in the MEL and placebo groups. Further, the occurrence of intraventricular hemorrhage (IVH), necrotizing enterocolitis (NEC), retinopathy of prematurity (ROP), and bronchopulmonary dysplasia (BPD) in all enrolled preterm newborns was analysed.

2.1. Procedures. Blood samples (0.5 mL) were collected, by vein puncture, from each newborns at 24 and 48 hours after administration of MEL. The samples were immediately centrifuged (RTM 1500, T 4°C, 10 min) to remove cells and obtain the supernatant, which was then separated into two different microtest tubes, one of which contained BHT (butylated hydroxytoluene), and stored at −80°C. The obtained samples were subsequently analysed to measure melatonin and OS biomarker (AOPP, F2-IsoPs, and NPBI) concentrations. Plasma melatonin concentrations were measured by high-performance liquid chromatography and mass spectrometry (MS/MS) (Agilent Technologies 1200 series system and an AB Sciex API 4000 triple-quadrupole mass spectrometer) according to the method of Wang et al. [17]. Markers of protein and lipid peroxidation were measured by AOPP and F2-Isopr. Spectrophotometry, tandem mass spectrometer coupled with HPLC, and HPLC-DAD system were used to analyse AOPP, F2-Isopr, and NPBI [18–20]. AOPP were measured using spectrophotometry on a microplate reader. The instruments were calibrated with chloramine-T solutions that absorb at 340 nm in the presence of potassium iodide [18]. The LC-MS/MS method of Casetta et al. [19] was followed for determination of F2-IsoPs. The method was centered around an API 4000 tandem mass spectrometer coupled with HPLC Agilent 1200 series, which includes a binary pump, a thermostated well-plate autosampler, and a column over. Chromatography separation was carried out at a temperature of 30°C by a mixture of an aqueous solution of acetic acid (Eluent 1) and acetonitrile (Eluent 2). For measurements, the tandem mass spectrometer ran in multiple reaction monitoring with the electroscopy source operating in negative ion mode and by exploiting the transitions $m/z353.3 > 193.2$ for F2 IsoPr and $357.3 > 197.2$ for the internal standard d4-8-iso-$PGF_{2\alpha}$. The method of Paffetti et al. [20] was followed for NPBI measurement with HPLC-DAD system (Agilent 1100 series). The method is based on preferential chelation of NPBI by a large excess of the low-affinity ligand disodium nitrilotriacetic acid. To separate NPBI, a two-step filtration procedure was used: (1) filtration through a 100 kDa Vecta-Spin Micro-Whatman ultracentrifuge filter and (2) filtration through a 20 kDa Vecta Spin Micro-Whatman

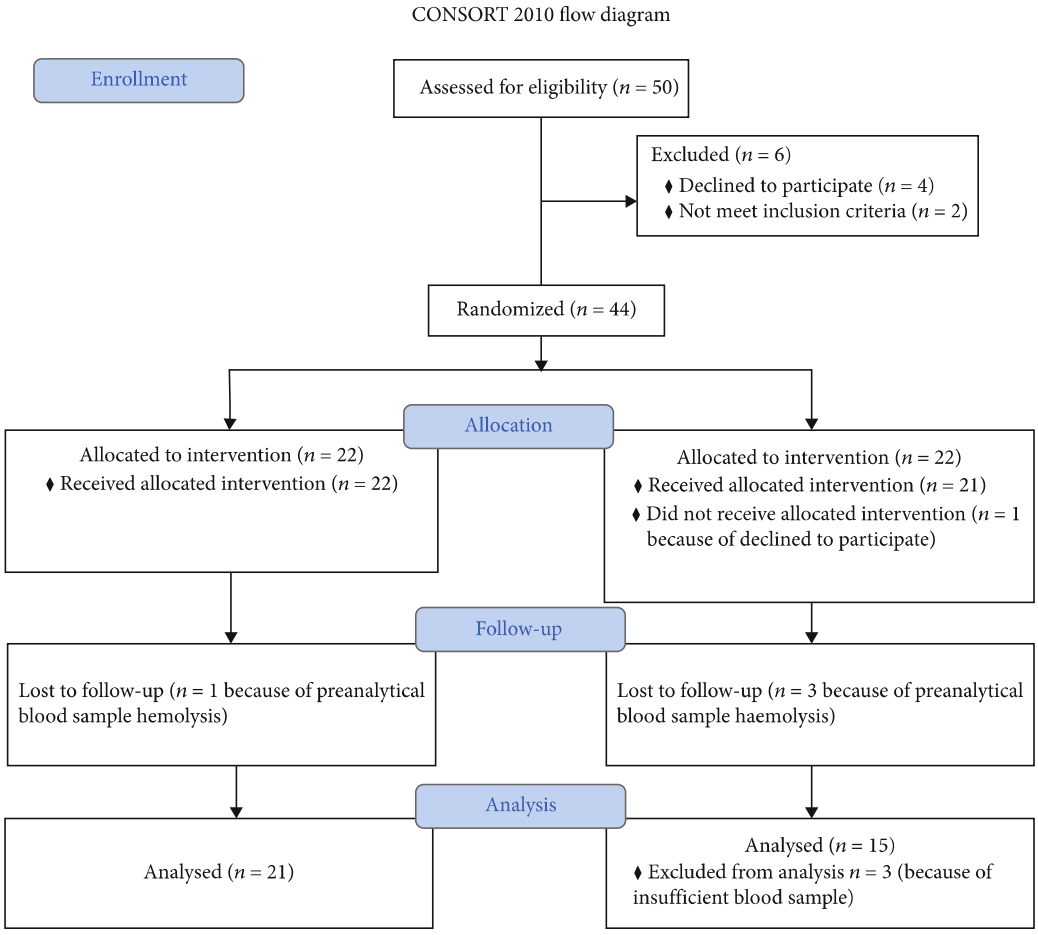

Figure 1: Consort diagram 2010.

ultracentrifuge filter at 13.660 × g and 4°C. The filtrate was injected directly into an isocratic reverse-phase liquid chromatography system using precolumn derivatization with the high-affinity iron ligand DHP, which forms a coloured complex with Fe3+ that absorbs at 450 nm. The analytic system detected iron as a ferric nitrate standard down to a concentration of 0.01 μM.

2.2. Statistical Analysis. A computer-generated randomization schedule was used to define the supplemented group (MEL group) or control (placebo group). Due to lacking data on oral melatonin supplementation in preterm newborns, sample size was calculated by G∗Power 3.9.1.2 for windows [21], estimating that between the 2 groups, there was a large difference in the concentration of melatonin (setting: effect size: 0.8, alfa error: 0.5, and power: 0.80); the minimum sample size required was 28. Statistical analysis was performed by SPSS version 25.0 for Windows (IBM, Armonk, NY, USA). Normal distribution of data was evaluated by Kolmogorov-Smirnov test. Data with non-normal distribution and categorical data were evaluated by Mann–Whitney U test and chi-square test, respectively. A p value < 0.05 was considered statistically significant.

Table 1: Clinical characteristics of enrolled population.

	MEL group ($n = 21$)	Placebo group ($n = 15$)	p value
Gestational age (wks)	32.26 ± 3.66	33.53 ± 2.88	NS
Birth weight (g)	1706 ± 638	1988 ± 513	NS
Gender (%)	$F = 14$ (66)	$F = 8$ (53)	NS
Spontaneous delivery (%)	5 (24)	2 (13)	NS
Caesarean section (%)	16 (76)	13 (87)	NS
NEC (%)	1 (4.7)	0	NS
BPD (%)	1 (4.7)	0	NS
IVH (all grade) (%)	4 (19)	3 (20)	NS

F: female; NEC: necrotizing enterocolitis; BPD: bronchopulmonary dysplasia; IVH: intraventricular hemorrhage; ROP: retinopathy of premature; NS: nonsignificant $p > 0.05$.

3. Results

The flow diagram of the study population from assessment for eligibility to analysis is reported in Figure 1. Out of the

Table 2: Melatonin, AOPP, NPBI, and F2-Isopr levels in test and control groups at 24 and at 48 hours of life.

	24 hours			48 hours		
	Placebo group Mean ± SD [median (25°-75°)]	MEL group Mean ± SD [median (25°-75°)]	*p* value	Placebo group Mean ± SD [median (25°-75°)]	MEL group Mean ± SD [median (25°-75°)]	*p* value
Melatonin (pg/mL)	28.57 ± 46.24 [10 (1-43)]	52759.30 ± 63529.09 [18309 (8886-100831)]	<0.001*	38.50 ± 44.01 [17 (4-121)]	279397.6 ± 516344.2 [37349 (10108-274844)]	<0.001*
NPBI (micromol/L)	2.40 ± 3.46 [0.7 (0.2-3.3)]	3.97 ± 3.13 [4 (1-6)]	0.113	2.99 ± 3.56 [0.8 (0.1-6)]	2.23 ± 2.37 [1 (0.6-5)]	0.525
AOPP (micromol/dL)	44.66 ± 26.54 [36 (28-45)]	36.07 ± 16.03 [32 (24-42)]	0.297	54.96 ± 24.33 [53 (33-75)]	51.66 ± 18.11 [47 (38-60)]	0.715
F2-Isoprostanes (pg/mL)	82.47 ± 51.30 [80 (31-121)]	75.05 ± 87.75 [46 (20-93)]	0.168	89.97 ± 52.01 [80 (62-127)]	36.48 ± 33.85 [24 (10-68)]	<0.008*

Data are expressed as mean ± SD and median (25-75°C).

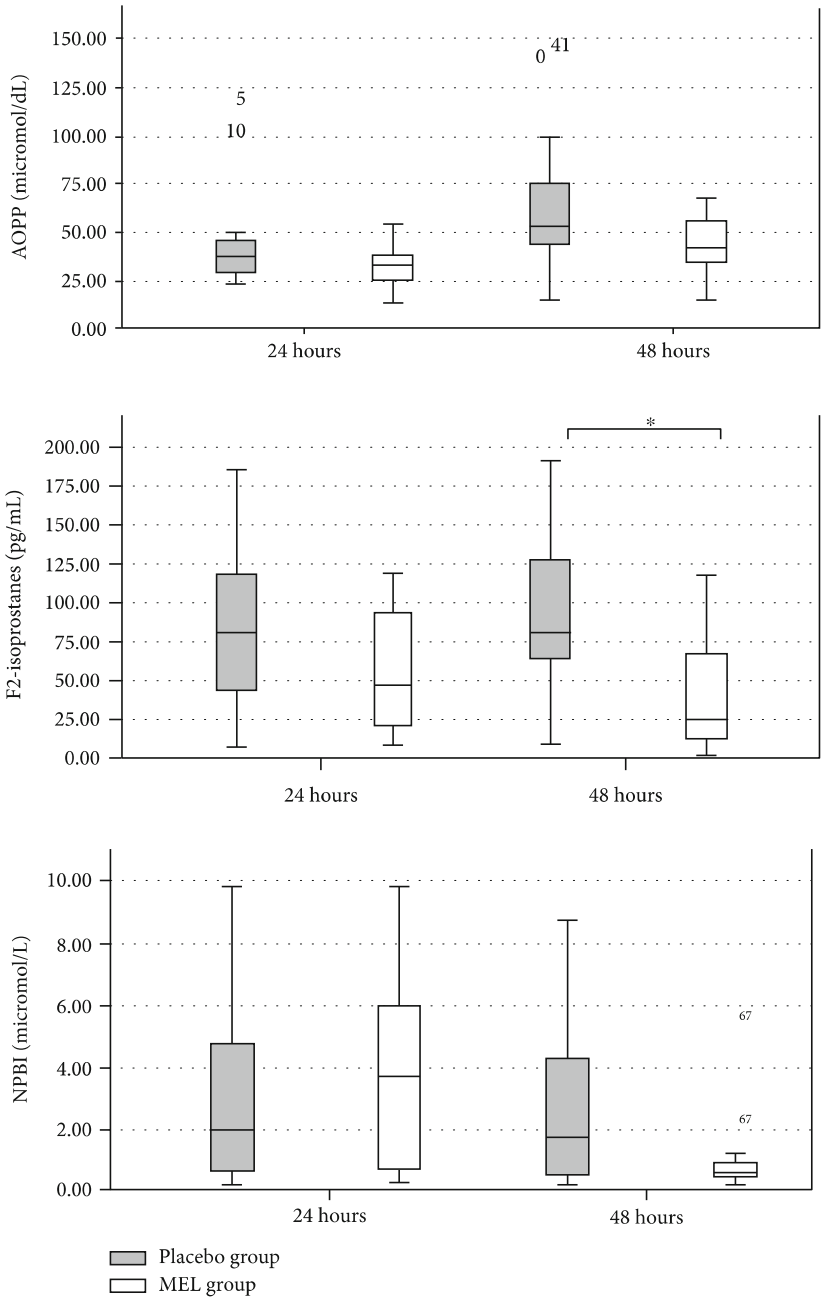

Figure 2: Isoprostanes, NPBI, and AOPP concentrations in placebo and MEL groups at 24 and 48 hours after melatonin administration. $^{*}p < 0.05$. Data are expressed as median (Q1-Q3). AOPP: advanced oxidative protein products; NPBI: non-protein-bound iron.

36 consecutively enrolled preterm newborns, 21 received melatonin (MEL group) and 15 received placebo (placebo group). Table 1 reports baseline characteristics of the enrolled population. Melatonin concentrations were significantly higher in the MEL group at 24 and 48 hours (Table 2). In the placebo group, male showed significantly higher concentrations of melatonin than female at 24 hours of life (58.1 ± 55.4 *vs.* 2.8 ± 3.5; $p = 0.001$); in the MEL group, female showed significantly higher concentrations of melatonin concentration than male at 48 hours of life (302296.3 ± 372402.9 *vs.* 22781.0 ± 35155.7; $p = 0.03$). No statistical difference between groups were found in AOPP and NPBI at 24 and 48 hours; also, F2-Isopr was not different at 24 hours (Table 2). At 48 hours, the mean plasma concentrations of F2-Isopr were significantly lower in the MEL group than in the placebo group (36.48 ± 33.85 *vs.* 89.97 ± 52.01 pg/mL, $p < 0.05$; Table 2, Figure 2). No differences between male and female in OS biomarkers were observed.

4. Discussion

The inability to counteract the harmful effects of free radicals is a matter of concern for all newborns, especially if preterm. The transition from intrauterine to extrauterine

environment is characterized by a huge of oxygen availability [11, 22]. This new hyperoxic condition increases the generation of various reactive oxygen species (ROS) such as hydrogen peroxide, singlet oxygen, and hydroxyl radicals that may attack macromolecules and cellular components. Moreover, ROS, as a secondary messenger, may trigger signalling pathways and induce stress-response genes or proteins [22, 23]. A significant increase in total hydroperoxides and AOPP levels from birth to 7 days of life has been reported in preterm newborns, indicating that damage caused from free radicals also occurs in nonhypoxic babies with normal clinical course [24]. Experimental studies in an animal model of hypoxic-ischemic brain damage report the effectiveness of antioxidant drugs to prevent or reduce ROS injury. Melatonin has been demonstrated to be able to block OS and inflammation pathways [25, 26]. In the first days of life, numerous factors could be responsible for an overproduction of free radicals, such as hypoxia, hyperoxia, acidosis, infections, transfusions, drug exposure, and pain [27]. Newborns are therefore peculiarly at high risk for OS-induced damage [28]. Therefore, there is compelling evidence that supplementation with antioxidant compounds may be effective in combating OS. Melatonin has not only free radical scavenging and antioxidant properties but also anti-inflammatory, antiapoptotic, and analgesic actions. Indeed, melatonin seems to modulate both pro- and anti-inflammatory cytokines in various pathophysiological situations wherein the balance between them determines the clinical outcome and to inhibit the expression of cyclooxygenase and inducible nitric oxide synthase, the nitric oxide production induced by lipopolysaccharide, and the inflammasome activation [11]. This fact is of clinical importance if we consider that inflammation is strictly related to OS in the pathogenesis of many diseases that affect preterm newborns [1]. Previous reports have suggested that preterm infants do not secrete melatonin until 52-week postconception [29]. In our study, we were able to measure the melatonin concentration in plasma of preterm infants who received placebo. All subjects received maternal or human donor milk which was a potential source of exogenous melatonin, being present in human milk [30]. Melatonin concentrations were found significantly higher in male than female in the placebo group at 24 hours of life and in female than male in the MEL group at 48 hours of life. To our knowledge, no data on melatonin differences between male and female have been reported. Immature hepatic metabolism and poor renal excretion may be responsible for a wider range of melatonin concentrations in treated preterm babies. Whatever the reason for the observed gender differences, the data should be checked in a large population due to the variability of melatonin concentrations in preterm newborn.

A protective effect of melatonin on lipoperoxidation was observed when orally administered in preterm newborns in the first days of life. Significantly lower levels of F2-Isopr were found in the MEL than the placebo group at 48 hours of life. This result is particularly important since early measurement of F2-Isopr has been recently described to discriminate patients showing abnormal white matter injury score at term of corrected gestational age with a cutoff value 31.8 pg/mL [31]. Moreover, high levels of urinary F2-Isopr were found in second days of life in newborns at high risk of developing a hemodynamically significant patent ductus arteriosus [32]. Increased levels of F2-Isopr have been also reported in preterm newborns affected by bronchopulmonary dysplasia or periventricular leukomalacia [33]. It was demonstrated that F2-Isopr provokes preoligodendrocyte death by oncosis, depending on inadequate antioxidant defences [34]. White matter injury, bronchopulmonary dysplasia, periventricular leukomalacia, and patent ductus arteriosus represent some of the peculiar diseases of prematurity, now grouped and called "free radical diseases of prematurity" because of the common pathways in pathogenesis [1]. The results of the present pilot prospective study show that few doses of melatonin decrease lipid peroxidation in preterm supplemented newborns. Thus, melatonin appears to reduce the risk of oxidative damage, protecting vulnerable organs and tissues in preterm newborns. F2-Isopr are the *in vivo* result of free radical-induced injury by peroxidation of lipids in cell membranes. They are stable compounds generated by the action of cyclooxygenase on long-chain unsaturated fatty acids. The mechanism involved in their formation implies that free radicals cause hydrogen abstraction from arachidonic acid and addition of molecular oxygen to form a peroxyl radical. F2-Isopr are terminal oxidation products with no further oxidant properties, therefore representing reliable markers of OS in newborns [35]. AOPP are the terminal products of the protein exposure to free radicals without oxidant properties, and they represent a marker of the degree of protein damage in oxidative stress conditions. We previously reported an increase of AOPP levels from birth to seventh day of life in preterm newborns [24]; in this paper, we observed a lower relative increment of AOPP level in treated newborns than controls. NPBI is a low-molecular-mass iron form, free from binding to plasma proteins. Iron toxicity derives from the production of hydroxyl radicals through the Fenton reaction. Thus, NPBI is a marker of potential OS because it indicates increased susceptibility to oxidative damage especially in vivo studies [35]. We previously found an association between NPBI and lipid oxidation in vitro [36]. In this study, no significant effect on NPBI and AOPP was observed at 24 and 48 hours from MEL administration, plausibly due to the small sample size associated with wide variability in biomarker plasma concentrations. Data could be also probably related to the multifactorial nature of the oxidative stress processes and to the need of higher doses of melatonin than those used. Furthermore, no significant effects were found on the prevalence of NEC, BPD, IVH, and ROP in the MEL group than the placebo group. It is noteworthy that the population study represented preterm newborns at medium-low risk to develop these diseases (mean gestational age > 28 weeks in both groups). Melatonin supplementation in extremely low birth weight or gestational age infants might have a major potentiality to reduce the increase of lipoprotein oxidation products. To our knowledge, lack of data exists regarding the valuation of melatonin efficacy in reducing term and preterm infant morbidity. This study has the limitation of few patients enrolled, and the results

need to be confirmed in larger trials. However, the results reported support for the first time the role of melatonin intake to protect preterm newborns against lipid peroxidation. The potential protective role of MEL is mainly due to its beneficial effect on plasma antioxidant status. Moreover, the safety profile of melatonin in clinical study is an encouraging start point for further investigate the protective effects of melatonin on organs and tissues. Our results pave the way for more medical research in this field before melatonin enters in clinical practice. Further research is needed as the schedule that might be effective and the subjects that might receive melatonin to obtain the greatest effect have not been precisely defined.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

Conflicts of Interest

The authors declare no conflict of interest.

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ANNEX 11

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Newborn metabolomic profile mirrors that of mother in pregnancy

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ABSTRACT

Background: Pregnancy is characterized by multiple metabolic processes to allow proper foetal development and ensure adequate stores. Little is known about the interactions between maternal and foetal metabolism during the last phase of pregnancy. Metabolomic offers potential to discover changes in maternal metabolism in pregnancy and their relation to the newborn metabolic status.
Objective: In this study we tested the hypothesis that metabolomic status in newborns at birth depends upon the metabolomic profile of their mothers in the last phase of pregnancy.
Study design: Urine samples were collected from 36 pregnant women three weeks before delivery and from 21 healthy term newborns within 48 h after birth. Urines were analysed using proton nuclear magnetic resonance (1H NMR) spectroscopy and NMR urine spectra were evaluated through Principal Components Analysis.
Results: The first component of the PCA analysis showed two distinct metabolic groups: pregnant women and newborns. A significant correlation was found between urine metabolic profiles of newborns and those of their mothers.
Conclusion: Urine metabolomic profiles of newborns at birth mirrors that of their mothers in the last phase of pregnancy. The metabolomic approach appears to be crucial to understand the maternal effects on foetal programming and infant outcomes.

Introduction

Pregnancy is characterized by a complexity of metabolic processes that may impact foetal development and infant health outcomes [1]. Understanding the changes in maternal metabolism before, during and after pregnancy is an essential clue regarding future neonatal health. In agreement with the "Barker hypothesis", there are many elements which may affect both the smooth progress of a pregnancy and the foetal and neonatal outcome [2–4]. Metabolomic is one of the techniques that best allows investigation of complex biological systems [5]. Metabolomic technology, measuring multiple metabolites, directly from biological systems, offers enormous potential to discover changes in maternal metabolism during pregnancy and their relation to the newborn metabolic status [6]. Recently, metabolomics has found a strong field of application in perinatology. Many studies have demonstrated that metabolomics is a powerful method for detecting detailed metabolic signature of healthy pregnancies adding an important step towards the identification of disease-related deviations of the major obstetric pathologies [7–9]. Diaz et al. observed a correlation between metabolomic profiles and foetal malformations [10]. Other authors investigated metabolomics in newborns from mothers with severe preeclampsia [11–13], in pregnancies with small for gestational age (SGA) foetus [7] and in pregnancies delivering preterm newborns [8]. Nevertheless, current understanding of the relationship between the metabolomic profile of mother and newborn during normal full-term pregnancy is still not complete. The present study aims to investigate the correlation between urinary metabolomic profile of healthy mother and their newborns. We tested the hypothesis that the newborn metabolomic status is associated to that one of its mothers, in the last trimester of pregnancy.

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Table 1
Clinical characteristics of pregnant women.

Gestational Age (weeks) at sample collection, mean (SD)	37 (1)
Mother's age (years) at delivery, mean (SD)	33 (4)
Weight gain (Kg) during pregnancy, mean (SD)	15 (5)
Maternal Gestational Diabetes, n (%)	4 (11)
Maternal Hypertension, n (%)	2 (6)
Maternal Hypotiroidism, n (%)	2 (6)
PROM, n (%)	9 (25)
Positive vaginal swab, n (%)	6 (17)
Type of delivery, n (%)	Vaginal 23 (64)C-Section* 13 (36)

Materials and methods

Population

The study was carried out at the Department of Molecular and Developmental Medicine, University of Siena, Italy. Institutional Review Board approved the study. A total of 57 urine samples were collected: 36 from pregnant women, three weeks before delivery, and 21 from healthy newborns, within 48 h of birth. Maternal and newborns' clinical details are reported in Tables 1 and 2. All of the urine samples were collected after written informed consent obtained from the individual women. The decision to use urine as the target of the investigations has been dictated by the need to make this study non-invasive and to facilitate adherence by patients. Regarding the mothers, the inclusion criteria were: -obtaining a free and informed consent from the pregnant women; -achievement of at least 36 weeks of gestational age (GA) at the time of urine sample collection; -time of delivery between 37 and 42 gestational age. The exclusion criteria were: -pregnancies with a gestational age < 37 weeks; -twin pregnancies.

Urine samples

Neonatal urines were collected by the insertion of a cotton pad inside the diaper. As regards to the collection of neonatal urine samples, no exclusion criteria were adopted if not the refusal to consent to the collection of the sample.

Each patient, pregnant women and newborns, provided a single sample during the study.

All urine samples were shipped in dry ice to the Laboratory of the University of Siena.

The samples were then analysed using a nuclear magnetic resonance spectroscopic (Nuclear Magnetic Resonance, NMR) analysis technique.

NMR analysis

Urine NMR measurements were performed on a Bruker DRX 600 MHz Avance Spectrometer with a selective inverse probe (SEI) equipped with Z gradient coil. Spectra were acquired at a constant temperature of 298.0 ± 0.1 K by using 90° pulses. Furthermore, 10 s delay was included in the pulse sequence to allow T1 relaxation. In fact, T1 values (in the range 1.5–2.8 s) of the analysed metabolites are such that a 10 s delay allows full recovery of longitudinal magnetization after a 90° pulse, as verified by constant integral values for D1 ≥ 5 s. A 0.3 Hz line broadening function was applied before Fourier transformation. A saturation pulse of 2 s duration was applied at the water resonance to suppress the water signal. 32 K data points per scan were used, and 128 transients were accumulated. Each urine sample was first centrifuged at 2000 rpm for 5 min and analysed afterwards. Sample (550 µl) plus 50 µl of a TSP-d4 20 mM solution were measured into a 0.5 mm (outer diameter) MR tube. All spectra were first run at their own physiological pH; we use this first spectrum only for an overview of the contained metabolites; then, we adjust the pH at 2.50 ± 0.02 in the same MR tube, with a microelectrode, and we run a second spectrum. The chemical shift of ionizable fluids is highly dependent on the pH. At a pH of 2.50, all chemical shift values are reproducible within ± 0.01 ppm [14]. Moreover, under the described conditions, the methyl signals of creatine and creatinine are clearly separated (3.05 ppm for the methyl signal of creatine and 3.13 ppm for creatinine) and the methyl signal of lactic acid (1.41 ppm) is not overlapped by the methyl resonance of threonine (1.33 ppm). The pH was adjusted using a minimal volume of HCl, starting from a 3 M and ending with a 0.05 M, and samples were directly frozen at −80 °C [15]. All samples were run at the same time. The variables of interest, related to the collected samples, were described in a dataset containing multiple clinical data of patients enrolled in the study.

Table 2
Clinical characteristics of newborns.

Gestational Age (weeks), mean (SD)	39 (1)	
Birth Weight (gr), mean (SD)	3370 (601)	
Small for Gestational Age, n (%)	–	
Large for Gestational Age, n (%)	4 (19)	
Gender, n (%)	12 Male (57)	9 Female (43)

Statistical analysis

The spectra were examined by analysis of the main components (PCA), through complex computer processing systems, based on anamnestic and clinical data (collected in the dataset) relating to both pregnant and newborn. The PCA is the first statistical approach to a metabolomic analysis and is aimed at finding a trajectory or a possible cluster formation in the study samples on the Cartesian plane, also called *score plot*. At first, a technique was used for data simplification, as the variables studied were multiple. This technique allows to obtain a linear transformation of the variables that projects the original ones into a new two-dimensional Cartesian system in which the new variable with the greatest variance in data is projected on the horizontal axis (values of the principal component 1, *PC 1*), while the second variable for variance size, called principal component 2 (*PC 2*) on the vertical axis. Then, a multivariate regression system was used, with the aim of making quantitative predictions relative to one or more properties of the spectra in question, using the *Partial Least Square* (PLS). PLS is a further development of the PCA, as the components used are derived from the set of PCA responses. In this way it is possible to maximize the variance not only of PC 1 (abscissa) but also of PC 2 (ordinate). In doing so, the choice of factors (main components), to be used for analysis, is even more focused and effective. The PLS allows to better balance the information contained in the abscissae and the ordinates on the *score plot*, reducing the effect of large but irrelevant variations between the data provided.

Results

Fig. 1 reports the median spectrum relative to maternal urine (Fig. 1A) and the median spectrum relative to neonatal urine (Fig. 1B). The first component of the PCA analysis (PC1) showed two distinct metabolic groups: pregnant women and newborns (Fig. 2). Among all enrolled samples (n = 57), 14 were pairs: a mother and her own baby. A significant correlation was found between urine metabolic profiles of mothers collected 3 weeks before delivery and those one of their newborns collected after birth, as shown through the scores of the second component (PC2) of the PCA analysis (Fig. 3). In Fig. 3 each square corresponds to a pair (mother-child) which a specific colour has been assigned within the scatter plot.

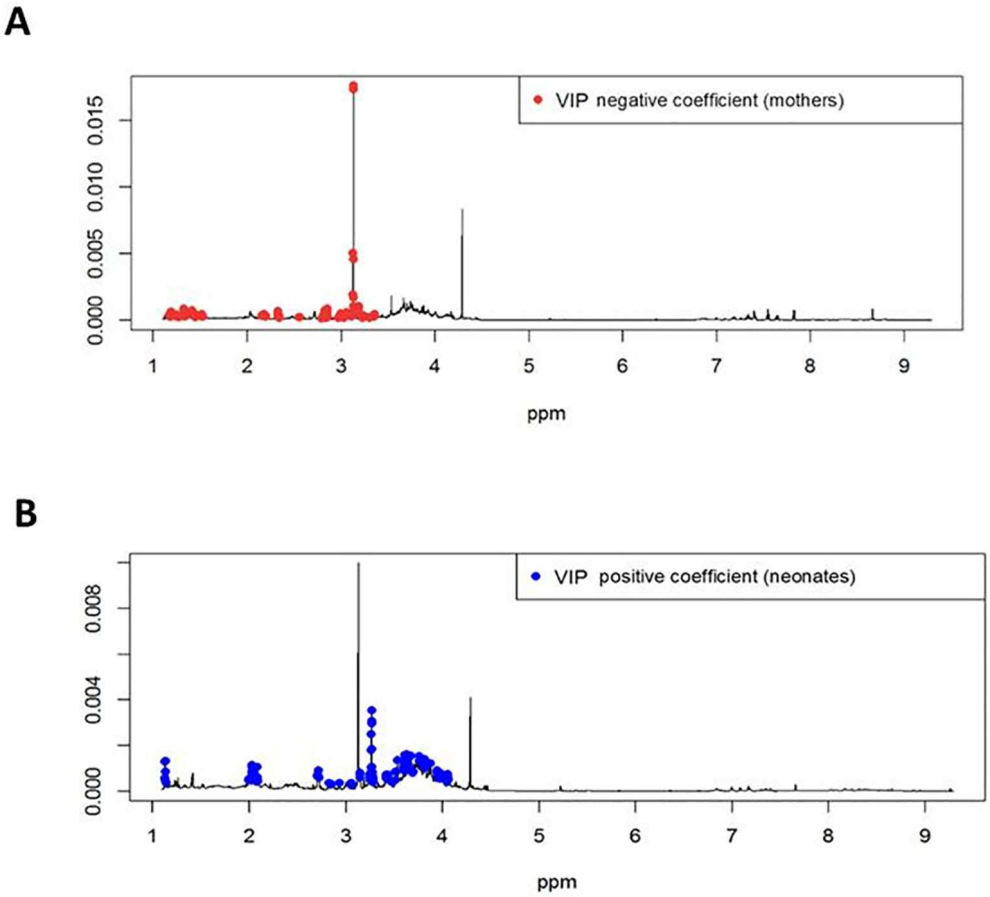

Fig. 1. Median spectrum of mothers (A) and newborns (B); VIP (Variable Influence in Projection), ppm (parts for million).

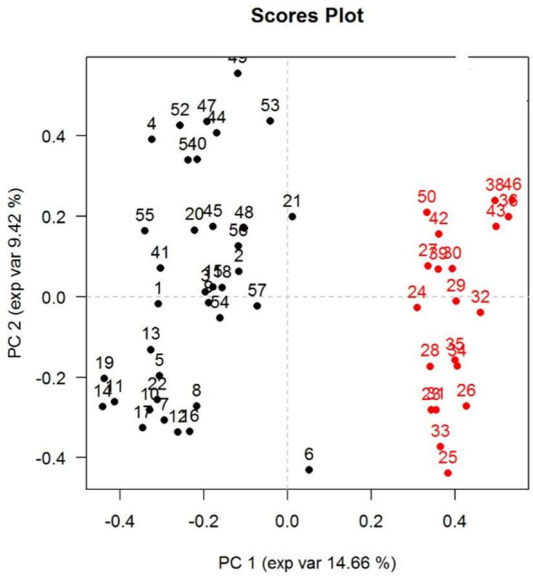

Fig. 2. The first component of the PCA analysis (PC1) of urine metabolic profile of pregnant women (black dots) and newborns (red dots). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

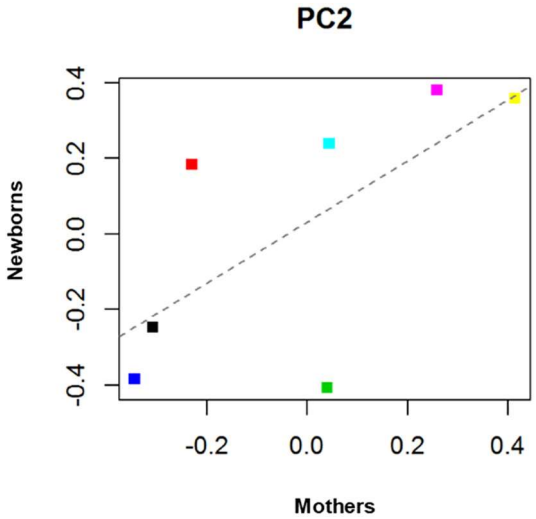

Fig. 3. Analysis of the 14 pairs: mothers and their own baby. Correlation between urine metabolic profiles of mothers collected 3 weeks before delivery and those one of their newborns collected after birth.

Discussion

Principal findings of the study

The principal findings of the study are as follows: 1) analysis of the urine spectra showed that the metabolic profile of pregnant womens is different from that of newborns, 2) the PCA analysis demonstrated that urinary metabolic profile of pregnant mother, three weeks before delivery significantly correlated with metabolic profile of their own babies, 3) there are several metabolites that help to highlight the correlation in the profiles of the mother and her child. These metabolites are a powerful link between the two profiles. The study provides, for the

first time, a comparison of the metabolomic profile of mother and newborn during normal full-term pregnancy. Already in pregnancy there is a bound between maternal and neonatal metabolism, despite substantial qualitative and quantitative differences between the two types of urinary profiles. Since metabolomic is sophisticated technique and they use highly complex data interpretation tools, the correlation found between the two types of profiles is strong and clearly visible [16]. If every newborn is a reflection of her mother, there will be much wider margins of clinical and therapeutic action on which to act. A first step could be identifying multiple metabolic profiles associated with physiological pregnancies, in order to create a metabolomic database that can enclose indicative cluster of a pregnancy free of complications. It is important to investigate whether the strong correlation between the two types of metabolomics profiles (mothers-newborns) can be quantified and highlighted from the very first stages of pregnancy, in order to foresee on eventual intrauterine abnormal development, even in early phases of pregnancy. Recently has been reported that maternal diet may influence offspring's health, even within well-nourished populations [17]. The knowledge that the newborn metabolic profile reflects the flux of nutrients and other metabolites between the maternal and placental-foetal unit is crucial, paving the way for future studies to understand the effects of maternal biochemistry, physiology and lifestyle behaviours on foetal programming and infant outcomes. Walejko et al. provided information on the metabolic profiles of maternal and foetal placental tissues delivered by caesarean section showing that there are different metabolic alterations in the maternal and foetal tissues of the placenta following delivery [18]. In the light of these results, our study on the “omics” appears paramount for a better understanding of the bond between the mother and the newborn. The in-depth knowledge of the metabolism of each pregnancy is very important, not only for a good state of maternal health, but also because we could prevent or intervene, in advance, in situations in which foetal well-being is at risk. This would represent, in the clinical field, a screening tool, relatively low-cost and non-invasive, for some disorders in pregnancy involving pathophysiological alterations of the same metabolism.

Strengths and limitations

The current study was the first to utilize metabolomics, a technology that provides highly discriminating power and sensitivity, to investigate the comparison of the metabolomic profile of mother and newborn during normal full-term pregnancy. The study is limited in that we didn’t know which were the metabolites that support the correlation between the mother and her child. However, the information presented here reveals that each newborn is a mirror of the metabolic environment of the womb.

Conclusions

The metabolic profile of newborn correlates with the maternal one at 3 weeks before delivery, suggesting that the newborn is plausibly “programmed” by the maternal metabolism and this happens, most likely, even in earlier phases of pregnancy. Since the newborn metabolic profile reflects the flux of nutrients and other metabolites between the maternal and placental-foetal unit, metabolomic approach appears to be crucial to understand the effects of maternal biochemistry, physiology and lifestyle behaviours on foetal programming and infant outcomes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mehy.2019.109543.

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ANNEX 12

Perrone, S.; Negro, S.; Laschi, E.; Calderisi, M.; Giordano, M.; De Bernardo, G.; Parigi, G.; Toni, A.L.; Esposito, S.; Buonocore, G. "Metabolomic Profile of Young Adults Born Preterm". *Metabolites* 2021, *11*, 697. https://doi.org/10.3390/metabo11100697

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Metabolomic Profile of Young Adults Born Preterm

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Abstract: Prematurity is a risk factor for the development of chronic adult diseases. Metabolomics can correlate the biochemical changes to a determined phenotype, obtaining real information about the state of health of a subject at that precise moment. Significative differences in the metabolomic profile of preterm newborns compared to those born at term have been already identified at birth. An observational case–control study was performed at the University Hospital of Siena. The aim was to evaluate and compare the metabolomic profiles of young adults born preterm to those born at term. Urinary samples were collected from 67 young adults (18–23 years old) born preterm (mean gestational age of 30 weeks, $n = 49$), and at term of pregnancy (mean gestational age of 38 weeks, $n = 18$). The urinary spectra of young adults born preterm was different from those born at term and resembled what was previously described at birth. The Random Forest algorithm gave the best classification (accuracy 82%) and indicated the following metabolites as responsible for the classification: citrate, CH2 creatinine, fumarate and hippurate. Urine spectra are promising tools for the early identification of neonates at risk of disease in adulthood and may provide insight into the pathogenesis and effects of fetal programming and infants' outcomes.

Keywords: preterm newborn; NMR spectroscopy; urine samples

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1. Introduction

Fetal and extrauterine life represents a continuum, during which the growth and development of the human being are influenced by genetic, environmental and social factors. Numerous studies have identified prematurity as a risk factor for the development of chronic adult diseases such as obesity, insulin resistance [1,2] and hypertension [3,4]. Recent evidence documents the fetal, rather than postnatal, origin of some chronic adult diseases. It is likely that fetal reprogramming occurs when the normal pattern of fetal development is disrupted by an abnormal stimulus or an "insult" during intrauterine life, which leads to adaptations by the fetus to allow for its survival, but could ultimately result in permanent structural and physiological changes with long-term consequences in adulthood. Early in utero life is vulnerable to perturbation, and compelling evidence indicates that the fetal period of development is extremely sensitive to environmental cues. Insufficient fetal substrates determine permanent structural and physiological changes, leading to long-lasting consequences in postnatal life [5,6]. Many experimental studies have been

conducted to explain the phenotypic consequences of fetal–placental perturbations that predispose individuals to the genesis of metabolic syndrome in adulthood. Metabolomics is an emerging omics science, considered today as the key for personalized medicine, able to correlate the biochemical changes (characterizing the organism of the human being, exposed to multiple intrinsic and extrinsic stresses) with a determined phenotype, and obtaining real information about the state of health of a subject at that precise moment [7]. Metabolomics has already identified significant differences at birth in the profile of preterm newborns compared to those born at term. Gracie et al. studied the importance and value of omics technologies and integrated them precisely for the study of preterm newborns [8]. Distinct metabolomic profiles were identified in infants born at different gestational ages, both in term and in preterm newborns [9], and in fetal growth-restricted infants [10]. However, very few studies have extended the follow-up of preterm infants into adult life [11–13]. The aim of our work is to identify and to compare the metabolomic profile of young adults born preterm to term controls, testing the hypothesis that metabolic profile in adulthood differs according to gestational age and resembles that of birth.

2. Results

One hundred and twenty-eight preterm newborns met the inclusion criteria. Among them, 23 were deceased at the time of enrolment and 32 were untraceable through the available contact details. Nine were ineligible according to the exclusion criteria and 15 denied consent to participate in the study. Nineteen young adults born at term in the same study period (years 1990–1997) were selected as controls. One of them denied consent while the study was underway (Figure 1).

Therefore, the final study population consisted of 67 young adults: 49 born preterm (18 females and 31 males; gestational age: 30.25 ± 2.7 weeks; birth weight: 1131.91 ± 118.15, current age: 21 ± 2.4 years) and 18 born at term (6 females and 12 males; gestational age: 38.5 ± 1.4 weeks; birth weight: 3120.43 ± 261.02; current age: 20.9 ± 2.5). For the clinical characteristics of the enrolled population, see Table 1.

Table 1. Perinatal and actual data in case and control groups.

Variables	Cases (n = 49)	Controls (n = 18)	p-Value
Maternal age (years), mean (SD)	31.19 (4.72)	31.15 (4.04)	Ns
Gestational age (weeks), mean (SD)	30.25 (2.72)	38.52 (1.44)	<0.05
Birth weight (grams), mean (SD)	1131.91 (118.15)	3120.43 (261.02)	<0.05
Male gender, n (%)	31 (63.26)	12 (66.6)	Ns
Apgar score at 1 min, median (IR)	5 (1–10)	9 (8–10)	<0.05
Apgar score at 5 min, median (IR)	8 (1–10)	10 (10–10)	<0.05
Neonatal resuscitation, n (%)	43 (87.7)	-	-
Intraventricular hemorrhage, n (%)	16 (32.6)	-	-
Hospital stay (months), mean (SD)	2.15 (1.11)	-	-
Age at assessment (years), mean (SD)	21.68 (2.42)	20.95 (2.55)	Ns
Caucasian population, n (%)	47 (95.9)	18 (100)	Ns
Same region of residency, n (%)	48 (97.9)	16 (88.8)	Ns
Actual mean systolic/diastolic blood pressure values (mmHg)	105/73	108/75	Ns
Actual body mass index < 18.5, n (%)	11 (22.4)	4 (22.2)	Ns
Actual body mass index 18.5–25, n (%)	34 (69.4)	13 (72)	Ns
Actual body mass index > 25, n (%)	4 (8.1)	1 (5.5)	Ns
Sport, n (%)	16 (32.6)	7 (38.9)	Ns

Ns: non significative.

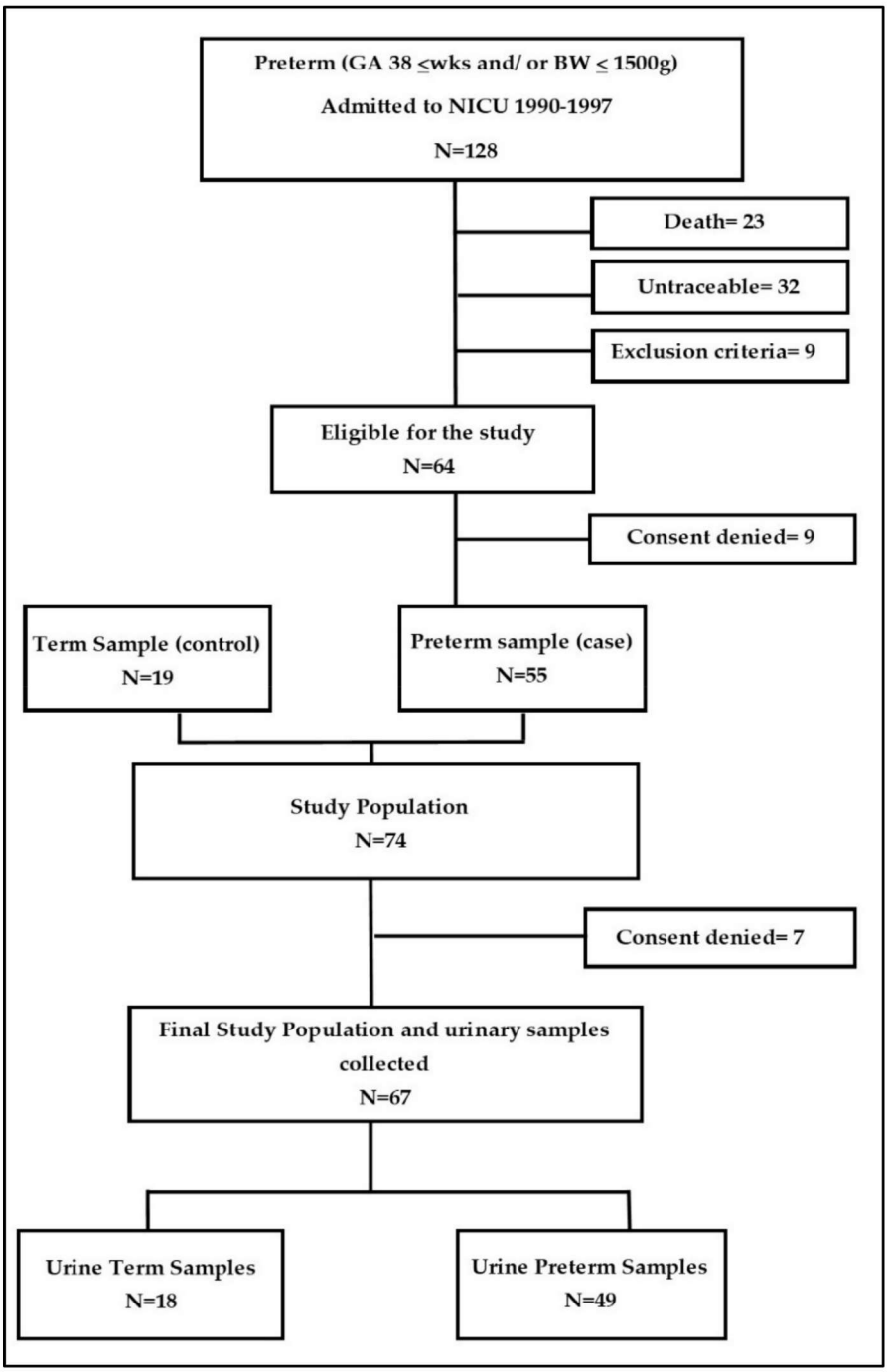

Figure 1. Participant flow chart; GA: gestational age; BW: body weight.

Multivariate (chemometric) analysis allowed us to highlight differences in the urine metabolomic profile between young adults born preterm and young adults born at term. A non-supervised Principal Component Analysis (PCA) technique was performed to find clusters within the data set. The PCA did not show a clear difference between "preterm" and "term" clusters (Figure 2).

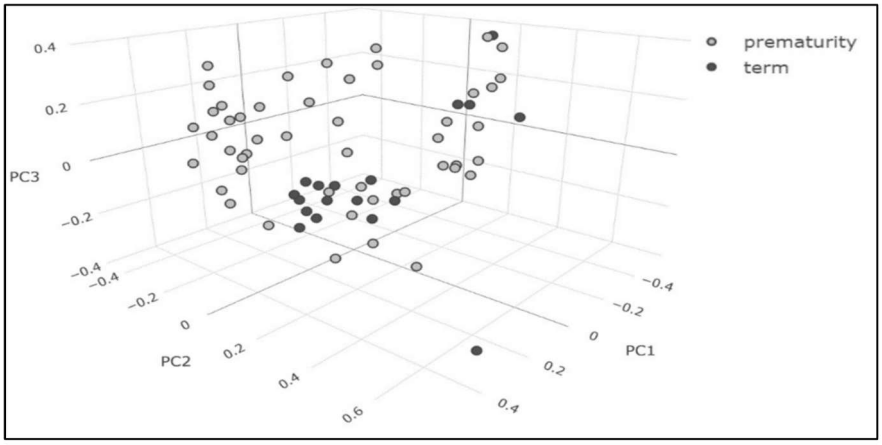

Figure 2. PCA score plot of the first three principal components; the two classes of patients, "preterm" and "term", are represented in light gray and black points, respectively; PC: principal component.

Therefore, the next supervised step by means of a supervised technique was required. Firstly, classification tasks were performed using nuclear magnetic resonance spectral data as the input for the classification models. A leave-one-out cross-validation technique was used as a resampling method to estimate the models' performance. The models, nevertheless, were not yet very discriminative (the accuracy, i.e., the percentage of patients correctly classified by the predictive algorithm, was about 70%, Table 2).

Table 2. Performance of the classification algorithms, obtained using scaled spectral data as input.

	Accuracy	F1 Measure	False Positive Rate	False Negative Rate	True Positive Rate	True Negative Rate
RF	0.7	0.82	0.94	0.06	0.94	0.06
GBM	0.72	0.82	0.72	0.12	0.88	0.28
SVM	0.73	0.84	1	0	1	0

RF: Random Forest; GBM: gradient boosting machine; SVM: support vector machine.

Therefore, classification tasks were performed using principal components as input data. The models' performances were estimated using 3 to 10 principal components to select the number of principal components with the best overall fit for each model (Figure 3).

The best classification result was obtained using the Random Forest (RF) model and the first three principal components as variables (accuracy ~82%). Moreover, to understand the contribution of each of the aforementioned components to the classification, an "importance" measure was computed through the RF algorithm. In order to identify the discriminating metabolites between preterm and term groups, the first three main components were then analyzed. The values of the loadings for each component were reported to understand how the metabolites contribute to each of the principal components (Figure 4).

Positive values of the loadings indicate that a variable and a principal component are positively correlated; negative values indicate a negative correlation. Large (either positive or negative) values of the loadings indicate that a variable has a strong effect on that principal component. Some thresholds were set to select the variables with the highest absolute loading values (Figure 5).

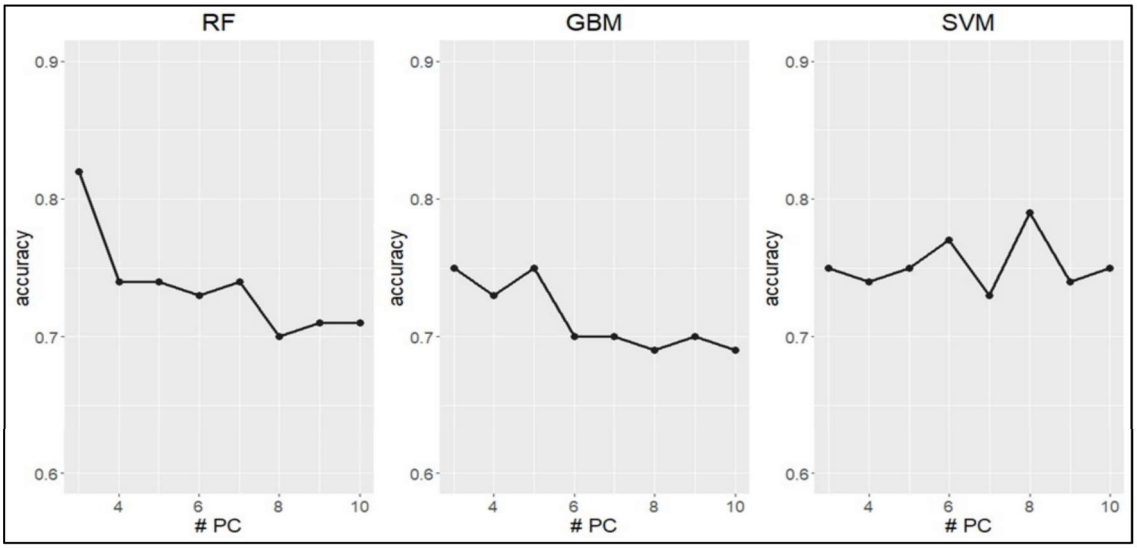

Figure 3. Accuracy of the models, estimated through the use of 3 to 10 PC as input data. RF: Random Forest; GBM: gradient boosting machine; SVM: support vector machine; PC: principal component.

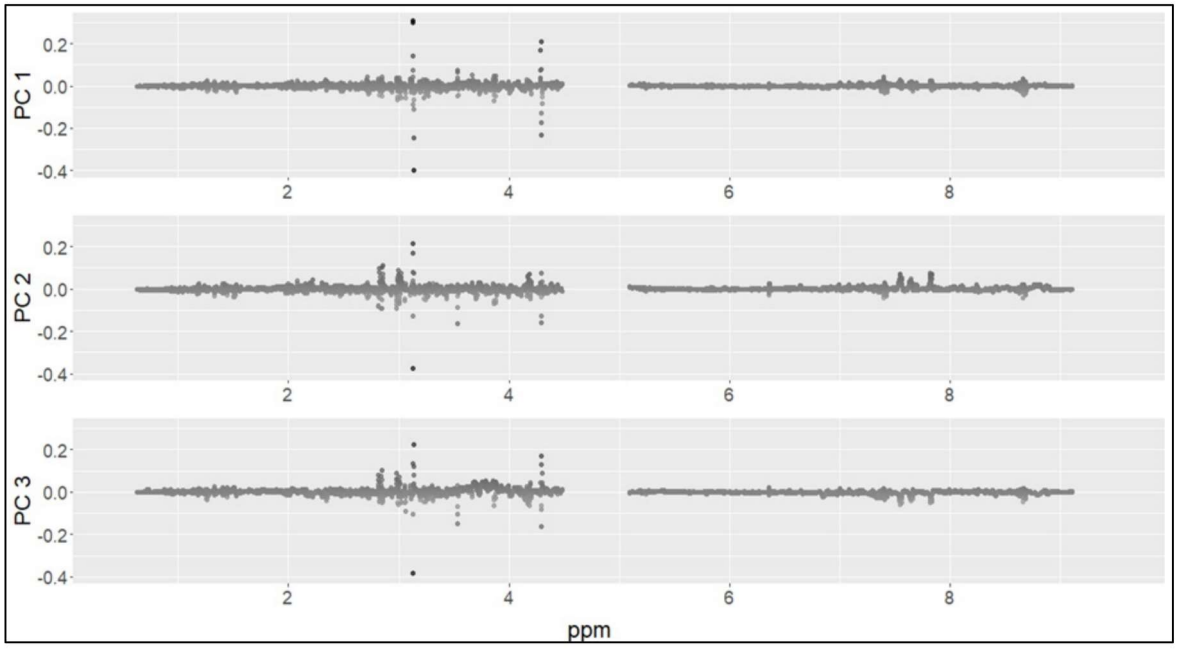

Figure 4. Values of the loadings of the first three principal components; the graphs illustrate which metabolite spectra (ppm) were most responsible for the "variance" in each of the three main components (i.e., the metabolite that has the higher absolute values).

The threshold values 0.1 and 0.05 were too high (only a few metabolites were selected for these values). For the threshold values 0.025 and 0.02, four ranges were identified in the ^{1}H-NMR (proton nuclear magnetic resonance spectroscopy) spectrum: 1.3–1.5 ppm, 2.7–4.3 ppm, 7.4–7.8 ppm and 8.6–8.7 ppm (Figures 6 and 7).

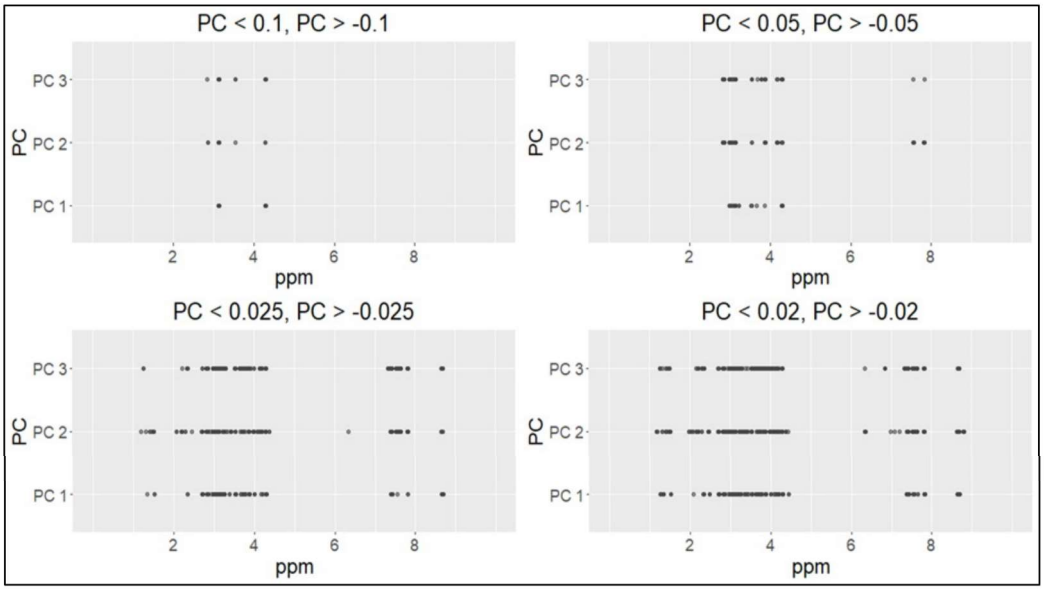

Figure 5. Different thresholds applied to the loadings of the first three principal components.

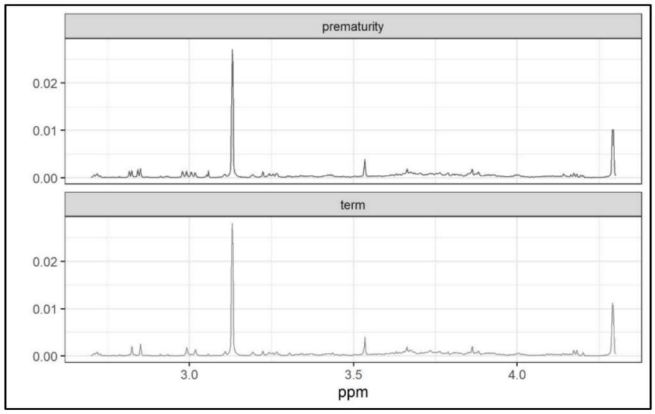

Figure 6. Comparison of mean spectra from each the two groups.

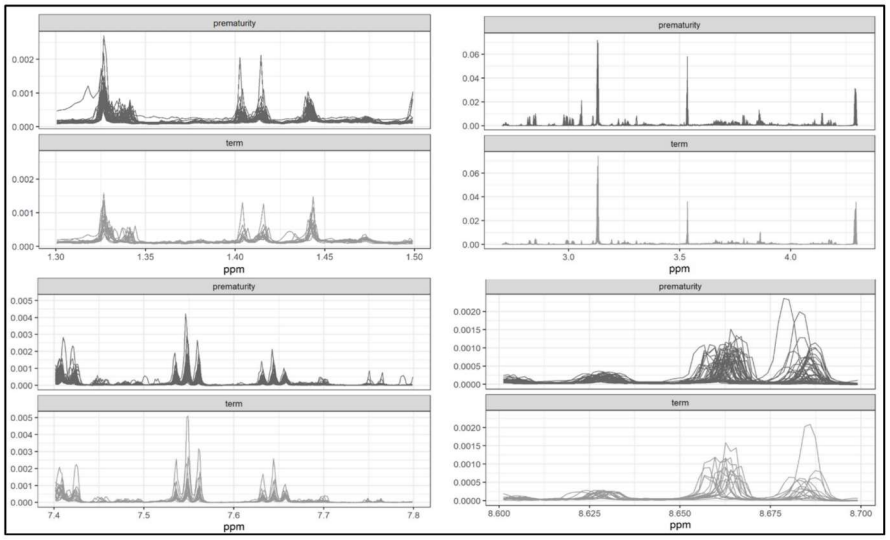

Figure 7. Spectra of the most important signals in the two groups.

The most significant signals, which distinguished the metabolome of preterm from that of term newborns, came from the following metabolites: citrate (3.13 ppm), CH2 creatinine (4.28 ppm), fumarate (6.8 ppm) and hippurate (7.6–7.8 ppm).

3. Discussion

The main finding of this research was that the urine metabolomic profile of adults born preterm significantly differed from the metabolic profile of adults born at term. With the unlabeled metabolomics approach, we were able to identify the significant spectra, which differentiated the two young adult populations (preterm vs. term). In particular, the involved metabolomic cycles most related to the characterizing metabolites found in the group of preterm (citrate, CH3 creatinine, CH2 creatinine, fumarate and hippurate) were tyrosine metabolism, tryptophan and phenylalanine biosynthesis, the urea cycle and arginine and proline metabolisms. Interestingly, these metabolomic patterns were the same as those found and described by Atzori L et al. in preterm newborns at birth, suggesting that the metabolomic profile of a young adult born preterm mirrors that of their perinatal period [9]. The same urine metabolites were also identified as influent in a recent study describing the variation in urine metabolites during the catch-up growth in the first months of life [14]. In this study, the authors found that hippurate and other metabolites were related to an individual's weight, while citric acid and creatinine were both related to a subject's weight and height. In the case of citrate, which is part of several pathways regulating carbohydrate, fat and protein metabolism, age-dependent concentrations have been reported in other metabolomic studies. Creatinine is the waste product of the energy muscle metabolism; it is constantly excreted through glomerular filtration, and its concentration in urine and in blood is routinely used as a marker of renal function. Creatinine urinary level appeared to increase with increasing age and body weight, following the increase in muscle metabolism that occurs during childhood and early adult life, with an increase in physical activities [14,15]. The finding that there are specific metabolomic patterns in young adults born preterm that mirror those found in the neonatal period and differ from those found in young adults born at term, confirm that biological samples have unique and distinctive biochemical compositions, which change in response to physiological (body weight, height and age) or pathophysiological stimuli (preterm birth). We hypothesized that an intrauterine environment that is not favorable for optimal embryonic and fetal growth may cause a placental and fetal "reprogramming" with changes in growth patterns and body metabolism that persist, unaltered, over the years [16]. Previous metabolomic studies performed in premature infants have already shown a difference in the levels of amino acids, enzymes and endocrinological markers collected from blood samples in the period immediately after birth (within 24–72 h from birth), showing that children at different stages of prematurity are metabolically distinct [17]. Moreover, it is already known that the adverse environment that preterm infants face during the preconceptual, fetal and postnatal period may have long-lasting effects on their adulthood health [18,19]. Therefore, the "snapshot" produced by the metabolomics provides fingerprinting of the state of health, useful for investigating the body's metabolomic responses to the disease and external stimuli [20]. Although we believe in the relevance of the link among prenatal environment, fetal growth and adulthood health status in the predictive role of metabolomics in perinatology, the data are too limited to draw definite conclusions regarding the use of metabolomic profiles in clinical practice. Potential confounders (such as dietary intake and hormonal status) should be analyzed in detail and will benefit from studies on a larger number of patients to identify the effect of environmental factors and comorbidities on the metabolomics spectra. In our population, we did not find an association with hypertension or obesity, and we were not able to identify biomarkers for the risk of chronic disease in adulthood. This study has the limitation of including a small number of term control young adults and this may have affected the results for the personal profiles. However, the study population was well defined, with no variability in respect to location, lifestyle and eating habits. Gender and the related hormonal differences may also have influenced

these results. This study therefore represents a preliminary phase, and a validation of our results in a new and larger cohort is necessary to check their reproducibility. Looking at the growing global incidence of chronic metabolic diseases, this research contributes to unveil the main routes of reciprocal linking between environmental factors and genetic susceptibility factors. Epigenetic modifications consequent to intrauterine environmental stimuli may persist long after the stimulus has ceased, providing a mechanism to explain the long-term consequences of acute exposures in early life. Metabolomics and ^{1}H-NMR allow the analysis of biofluids or tissues to extract latent information and enable sample classification and biomarker identification. Although plasma, serum, amniotic fluid, cord blood or stool can be used for metabolomic analysis, urine samples, due to their non-invasive method of collection, are a very promising tool in the pediatrics and neonatology field. The future goal will be to identify more accurately patients at risk for chronic adult diseases, for which an individual therapeutic approach might be necessary.

4. Materials and Methods

An observational case–control monocentric study was carried out at the University Hospital of Siena, in the Neonatology–Pediatrics Unit. The urinary samples were collected from young adults recruited in the research study, "Multidisciplinary long-term follow-up of premature births: AOUS case series 1990–1997". They were enrolled to take part in the multidisciplinary follow-up study that was conducted at the University Hospital of Siena.

4.1. Inclusion and Exclusion Criteria

The study population was enrolled starting from a cohort of young adults born with gestational age (GA) $\leq$ 33 weeks and/or birth weight $\leq$ 1500 g, admitted to the Neonatal Intensive Care Unit at the Santa Maria alle Scotte Hospital, in the period between 1 January 1995 and 31 December 1997. Babies born at term in the same study period (years 1995–1997) were selected as controls (for the clinical characteristics of the enrolled population, see Table 1). Subjects suffering from genetic or malformative syndromes, inborn errors of metabolism, severe motor disability and all whose conditions prevented the completion of the performance-expected tests, were excluded from the study. Vegan or vegetarian diet and alcohol use also represented exclusion criteria. The study was conducted in accordance with the ethical principles enshrined in the Helsinki Declaration's latest revision. Patients eligible for the study were contacted by telephone and informed about the aims and methods of carrying out the study. Adherence to the study was voluntary. Nevertheless, official participation in the study was subject to the signing of an informed written consent, which guaranteed all rights regarding the protection of personal data according to the national law.

4.2. Clinical Data Collection

Eligible patients were invited to the Neonatology–Pediatrics Unit, Neurodevelopmental Follow-up Division. For each patient, we drew up a clinical folder, consisting of: a signed copy of the informed consent; data relating to the perinatal age retrospectively collected from the birth medical records (such as gestational age, birth weight, type of delivery, length of hospital stay at birth, complications or problems related to prematurity that came out during hospitalization and diagnosis at discharge); data related to the current state of health of the patient; the anthropometric parameters (including height, weight and body mass index achieved); and the clinical examination.

4.3. ^{1}H-NMR

Urine samples were collected and shipped in dry ice to the Laboratory of the University of Siena. The samples were then analyzed using the ^{1}H-NMR analysis technique. ^{1}H-NMR measurements were performed on a Bruker DRX600 MHz Avance Spectrometer with a selective inverse probe equipped with a Z-gradient coil, as previously described [21]. Briefly, spectra were acquired at a constant temperature of 298.0 $\pm$ 0.1 K using a 90° pulse.

A delay of 10 s was included in the pulse sequence to obtain the relaxation time T1. In fact, the values of T1 (in the range 1.5–2.8 s) of the considered metabolites were such that a delay of 10 s allowed for the full recovery of the longitudinal magnetization after a 90° pulse, as verified by integral values constant for D1 $\geq$ 5 s. A saturation pulse of 2 s suppressed the water signal during the water resonance. A total of 32 k data points per scan were used and 128 transients were accumulated. Each urine sample was measured after centrifugation occurred, 2000 ppm for 5 min. The pH of the urine samples was checked with a buffer solution (pH 7.4) containing trimethylsilylpropanoic acid (TSP). Samples (550 µL) plus 50 µL of TSP-d4 20 mM solution were measured into the 0.5 mm tube (tube diameter) of the ^{1}H-NMR. All ^{1}H-NMR spectra were first performed at their physiological pH. This first spectrum was used only to obtain an overview of the metabolites contained. A second spectrum was executed at pH 1.0 $\pm$ 0.02 in the same MR tube, with a microelectrode. The chemical shift of ionizable fluids is highly dependent on the pH. At a pH of 1.0, all chemical shift values were reproducible within $\pm$ 0.01 ppm. Spectra were aligned to compensate for the shift of the signals of some metabolites, due to small inter-sample pH changes. Then, they were uniformly binned to 0.0025 ppm intervals between 0.5 and 9.5 ppm, excluding the region corresponding to water (4.6–5.2 ppm) and TSP ($-$0.5–0.5 ppm) signals. Bins were normalized to the total spectral area to compensate for the different dilutions of original urine samples. To identify the most discriminating parts of the spectrum, the results of the classification algorithm were combined with the profiles of the respective loadings. A system of thresholds, defined empirically, then allowed the selection of the characteristic parts of the spectrum of the two groups.

4.4. Statistical Analysis

The data were analyzed using the R program (R Core Team (2016). R: a language and environment for statistical computing R Foundation for Statistical Computing, Vienna, Austria. Available online: https://www.r-project.org (accessed on 5 October 2021)). The data of sample characteristics with a normal distribution were evaluated by unpaired t-student test, while categorical data were analyzed by chi-square test. The study was conducted according to the classical metabolomic approach divided into two steps: an unsupervised and a supervised phase [22]. In order to find clustering evidence, a non-supervised technique (PCA) was performed on mean-centered and Pareto-scaled methods data. PCA was also used to detect possible outliers within the dataset. The next supervised step allowed us to model data through different classification systems: RF, support vector machine and gradient boosting machine. These different machine-learning algorithms were used to analyze the differences in the metabolomic profile that were connected to the different gestational age at birth [23–25]. As the classification algorithms employed do not provide direct methods for calculating the significance of the variables responsible for classification, alternative methods were used to define which elements could support the performance of the model. To select and identify metabolites that distinguish young adults born preterm from those born at term, a threshold method was used. By varying the threshold of interest, it is possible to look for the metabolites best expressed by the various principal components, and to estimate which are the most important for defining the classification. A threshold method allowed us to combine the rigor of a systematic approach (choice of classification model and identification of the most important principal component) with a more manual approach to test and choose the selection thresholds. It also allowed us to see if metabolites emerge, establishing a significance of the effects.

5. Conclusions

Urinary spectra were able to discriminate the metabolomic profiles of young adults born preterm from those born at term, revealing differences similar to those already reported at birth. Urine spectra may provide insight into the peculiar metabolomics of preterm babies that persists into adulthood, paving the way for further research on the pathogenesis and effects of fetal programming on infants' outcomes. This work is

preliminary research that opens the interest of neonatologists to the fingerprinting of prematurity. In-depth knowledge of the metabolomics of preterm babies is very important, not only for a good state of childhood health, but also because we could prevent or intervene, in advance, in situations in which neonatal development is at risk to become poor. This would represent, in the clinical setting, a relatively inexpensive and non-invasive screening tool for some early-life pathologies that involve pathophysiological alterations of the metabolism itself.

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