UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Salute della Donna e del Bambino

Corso di Dottorato di Ricerca in Medicina dello Sviluppo e Scienze della Programmazione Sanitaria

CURRICOLO: EMATO-ONCOLOGIA, GENETICA, MALATTIE RARE E MEDICINA PREDITTIVA

CICLO 34°

METABOLOMICS APPLIED TO BIOMARKER DISCOVERY IN RESPIRATORY OUTCOME OF PRETERM INFANTS

Coordinatore: Ch.mo Prof. Gianni Bisogno

Supervisore: Ch.mo Prof. Eugenio Baraldi

Dottorando: Dott.ssa Serena Calgaro

TABLE OF CONTENTS

ABSTRACT

Background: Bronchopulmonary dysplasia (BPD) is the most common chronic lung disease in preterm infants. Despite significant improvements in neonatal intensive care, its incidence is still increasing. The long-term respiratory and neurological consequences of BPD have a major impact on survivors' quality of life. Currently, there is still no specific treatment once BPD is established, so it is important to identify children at risk in the first days of life. Metabolomics is the most recent "omics" science and allows the identification and quantification of all metabolites present in a complex biological system. The application of the metabolomic approach to diseases permits the identification of characterizing metabolites, even unknown ones, which help to understand the pathogenetic mechanisms underlying the disease and potentially can become a diagnostic tool.

Aim of the study: The aim of the study is to apply the metabolomic approach in order to establish whether a biomarker profile exists at birth, capable of predicting the development of BPD in preterm infants. The secondary aim is to characterized the urinary metabolome of BPD patients at birth.

Materials and Methods: This is a monocentric prospective observational case-control study. It was conducted at the Neonatal Intensive Care Unit (NICU), Hospital-University of Padova, Padova, Italy. Premature neonates admitted to the NICU, born at less than 32 weeks' gestational age (GA) have been enrolled. Patients who develop BPD were enrolled as cases, patients without BPD as controls. BPD was defined as need for oxygen supplementation for at least 28 days. Urine sample was collected within 48 hours of life for each patient and untargeted metabolic analysis was performed with ultra performance liquid chromatography system coupled to a Quadrupole Time-of-Flight mass spectrometer.

Results: 161 neonates have been enrolled, of that 69 with BPD (42.9%) and 92 without BPD (57.1%). The median GA at birth was 29 (27-31) weeks and the median birth weight 1115 (800-1400) g. Urine untargeted metabolomic revealed a metabolites clustering in BPD neonates in comparison to neonates without BPD. The metabolomic derangements concerned metabolites belonging to the acylcarnitine class, uridine and pseudouridine. To control the effect GA on metabolome, 11 infants with BPD were matched with 11 without BPD of comparable GA. After adjusting the analysis for GA, the main discriminating metabolites were: L-cystathionine, isovalerylcarnitine, creatine, p-hydroxyphenylacetic acid. Compared to clinical data alone, the application of urinary metabolome

analysis at birth increases the sensitivity and specificity in the prediction of BPD, respectively from 86.7% to 90.0% and from 84.0% to 86.0%.

Conclusions: The metabolomic approach on urine collected within 48 hours of life revealed an interesting clustering of metabolites that discriminates between preterm infants at risk of developing BPD versus infants who will not develop it. The analysis of the identified metabolites at birth increases the prediction of BPD development in preterm infants. In the next future, early detection of infants at risk of developing BPD may be useful for the administration of targeted therapies.

BACKGROUND

Bronchopulmonary dysplasia (BPD) is the most common chronic lung disease in infants born preterm. [1] It was firstly described in 1967 [2] and, since then, the definition, the physiopathology and the epidemiology have continuously evolved, in relation to the advance in neonatal intensive care and the increasing in neonatal survival.

From the “old” BPD to the “new” BPD

Northway was the first who described a severe lung disease in preterm infants with respiratory distress syndrome at birth, treated with mechanical ventilation and high oxygen concentrations which he named bronchopulmonary dysplasia. [2] It was characterized by radiographic alterations with alternations of areas of hyperinflation and areas of increased density. On the histological level, BPD was characterized by emphysematous alveoli, marked hypertrophy of peribronchiolar smooth muscle, atelectatic areas associated with ordinary-appearing areas, alveolar epithelial cell heterotypical, perimucosal fibrosis, generalized focal thickening of basement membranes with marked separation of capillaries from alveolar epithelia, tortuous lymphatics and early vascular lesions of the pulmonary hypertensive type. [2]

In the last decades the improvement in neonatal intensive care, including gentler ventilatory strategies, the use of antenatal steroids and surfactant therapy has led on the one hand to a reduction in the development of BPD in preterm infants with more advanced gestational age (GA) and, on the other hand, to the survival of premature babies of lower GA, also with birth weight <1000 g. It is mainly these extremely low-birth weight infants who survive to develop the disease to date, with an overall increasing incidence trend of BPD over the years. [3] These changes have also led to a modification in the physiopathology and in the histopathological characteristics of BPD, now called "new" bronchopulmonary dysplasia, as opposed to the “old” BPD described by Northway. In these extremely low-birth weight infants the lung is just beginning to develop into a gas exchange organ. [4] So the major cause of lung injury is an interference with the normal lung development to which pre and postnatal factors act. Indeed the histopathology of infants’ lungs who died from “new” BPD showed that the saccular lung seems to have an arrest in alveolar and vascular development corresponding to the gestational age of the infant at delivery. The "new" BPD is characterized by fewer, larger and simplified alveoli associated with dysmorphic vasculature. Lungs have more

uniform inflation, less fibrosis and less alternation of emphysematous and atelectatic areas. Airway smooth muscle is only mildly thickened. [5]

Epidemiology

As mentioned above, in the last decades the incidence of BPD has raised. In infants born <29 weeks' GA and with birth weight <1500 g, the incidence of BPD rose up from 32% to 47% from 1993 to 2012 (Figure 1). The incidence of BPD is inversely related to GA, with higher incidence as lower gestational weeks at birth are. [3]

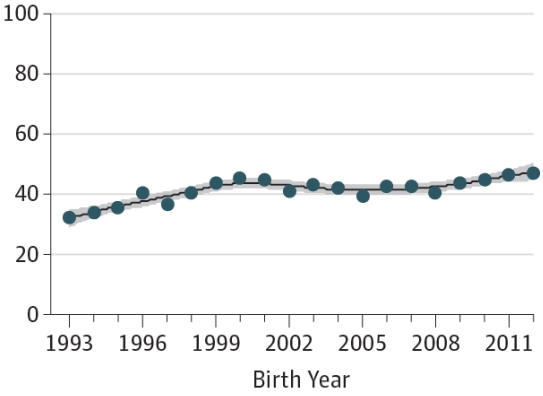

Figure 1. Incidence (%) of BPD from 1993 to 2012 in newborn <29 weeks' gestational age and birth weight <1500 g. [3]

However the trend over the years shows some differences in relation to the GA, with a greater increase in the incidence in the lower GAs: from 1993 to 2012 it rose up from 59% to 89% in infants born at 23 weeks' GA, while it rose up from 22% to 24% in those born at 28 weeks' GA (Figure 2). Even if infants born at >28 weeks' GA have lower incidence of BPD, the increase in survival of infants of birth weight < 1000 g which have higher incidence of BPD resulted in an overall increase in the incidence of BPD over the years. [3]

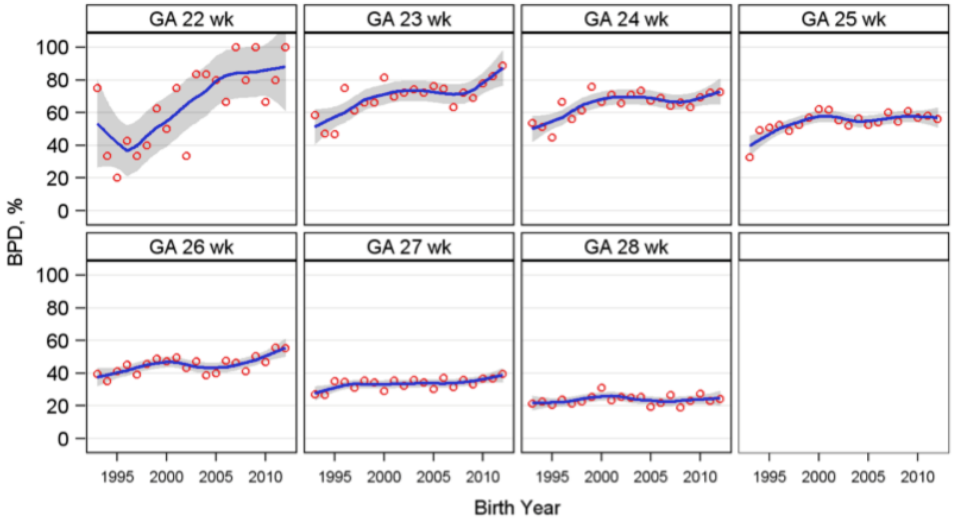

Figure 2. Incidence (%) of BPD from 1993 to 2012 in newborn at different weeks' gestational age at birth. [3]

Moreover, the incidence of BPD differs between levels of hospitals classified according to the American Academy of Pediatrics (AAP), with level III and level IV having lower BPD rates than level II

Neonatal Intensive Care Units (NICU). In particular, Lapcharoensap et al. reported an adjusted BPD rates of 50.3%, 46.1% and 47.7% for levels II, III and IV respectively and the minimum variation was observed among level IV NICUs. [6] This suggests that specific hospital environments and clinical practices affect the development of BPD. [7]

A recent systematic review on global incidence of BPD showed a wide range of incidence, ranged from 10% to 89% (Table 1). Also in this case, authors explained this great variability with differences in birthweight of infants included in the studies, as well as differences in diagnostic criteria used and intensive care practices over the continents. [8]

Table 1. Reported incidence of BPD in preterm infants <28 weeks' GA by

	Incidence range (number of distinct data sources)	
Region	All sources, % (*n*)	Population-based studies, % (*n*)
Europe	10–73 (23)	17–73 (8)
North America	18–89 (19)	18–75[a] (7)
Asia	18–82 (16)	25–56 (3)
Oceania	30–62 (6)	30–58 (3)
Africa	No publications found	No publications found
South America	No publications found	No publications found

Definition and diagnosis

Defining BPD is still challenging. Up to now various definitions of BPD have been proposed, reflecting the evolution of the disease and the new therapeutic strategies which require the continuous updating of diagnostic criteria.

The first who described BPD was Northway in 1967. [2] He defined BPD as a chronic pulmonary disease appeared in survival infants with acute respiratory distress syndrome at birth, after exposure to high oxygen concentration, characterized by typical radiographic and histological alterations. Chest x-ray showed rounded lucent areas in the lungs alternating with thinner strands of radiodensity. Cardiomegaly could be present. The autopsy showed in the lungs groups of emphysematous alveoli associated with bronchioles with marked hypertrophy of peribronchial smooth muscle. [2]

Tooley in 1979 defined BPD as a "respiratory insufficiency (hypoxemia and hypercapnia), accompanied by intercostal retractions, expiratory grunting and chest radiograph showing military

atelectasis or opacification". [9] He used the following diagnostic criteria: radiologic abnormalities of the lung at 30 days of life plus at least one of these:

- Arterial $paO_2 \leq 60$ mmHg of an infant breathing room air
- Arterial $paCO_2 > 45$ mmHg
- O_2 dependence.

In a Workshop on BPD of the National Institutes of Health (NIH) in 1979, BPD was defined as 28 days of oxygen therapy with radiographic changes. [5]

Shennan et al. in 1988 demonstrated that O2 dependence at 28 days of life could predict pulmonary abnormalities at 2 years follow-up only in 38% of infants, while 31% of those who were still receiving oxygen at 28 days of life did not develop impairment in pulmonary function at follow-up. [10] The need for oxygen at 28 days was a good predictor of abnormal findings in infants of 30 weeks' GA or more but it became increasingly less useful as GA decreased. The study showed that the need for oxygen at 36 weeks' post menstrual age (PMA) was a better predictor of distant lung outcome, regardless of GA at birth, with a positive predictive value of 63%. Therefore, due to the greater predictive capacity of pulmonary outcome, Shennan proposed oxygen dependence at 36 weeks' PMA as a diagnostic criteria for BPD. [10]

The NIH 2001 definition diagnoses BPD if the need for oxygen has been required for at least 28 days. The severity of the disease is classified in "mild", "moderate" or "severe" at 36 weeks' PMA or discharge (whichever comes first) for infants born at <32 weeks' GA or day of life (DOL) 56 for infants ≥32 weeks' GA, according to respiratory support needed (Table 2). [11] Treatment with oxygen and/or positive pressure should not reflect an "acute" event but the infant's usual situation.

Table 2. Diagnostic criteria of BPD according to NIH 2001 definition. [11]
Definition of abbreviations: NCPAP=nasal continuous airway pressure; PPV=positive-pressure ventilation.

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

Walsh et al. in 2004 proposed a physiological definition of BPD, based on oxygen administration, ventilatory support and assessment of saturation. [12] They were diagnosed with BPD infants of 35-37 weeks' PMA in mechanical ventilation, continuous positive airway pressure or oxygen administration with FiO_2 ≥ 0.3 and with oxygen saturation of 90-96%. Infants with oxygen administration with FiO_2 < 0.3 with oxygen saturation 90-96% or FiO_2 ≥ 0.3 and oxygen saturation >96% underwent an oxygen reduction test: infants who do not pass the test were diagnosed with BPD. No BPD were defined infants breathing room air with an oxygen saturation ≥ 90% or infants who pass the oxygen reduction test. [12] Using this definition, in comparison with Shennan' one, a reduction in the incidence of BPD was observed. There was also a reduction in the variability of incidence among centers, although the range of variability remained high.

With the evolution of therapeutic strategies, including the introduction of high flow nasal cannula (HFNC) into clinical practice, the diagnostic criteria of BPD proposed so far left some patients unclassifiable. In particular, in a multicentric observational study it was found that infants remained unclassified in 11% of cases with Shennan definition, 2.1% with NIH definition and 16.1% with the physiological definition. [13] Moreover, they have also potential reasons for misclassification:

- for Shennan definition: infants with positive pressure or HFNC with room-air were assigned to no-BPD group;

- for NIH definition: significant respiratory support at 36 weeks' PMA but less than 28 days of supplemental oxygen, nasal cannula flow was not considered positive pressure;

- for physiological definition: failure due to inability to wean off flow may have a different pathophysiology than failure to wean off oxygen. [13]

In consideration of all these limitations, a workshop sponsored by the National Institute for Child Health and Human Development (NICHD) was held in 2018 to review the definition of BPD. [14]

Patients supported with HFNC and infants who die from respiratory failure prior to 36 weeks' PMA were included in the definition. Supplemental oxygen need for 28 days was removed as it doesn't have good positive predictive value for long term outcomes and it is often misinterpreted as need for oxygen on DOL 28. Infants with persistent radiographic alterations were classified according to the therapy required to maintain oxygen saturation at 90-95% at 36 weeks' PMA (Table 3). [14]

Table 3. Diagnostic criteria of BPD according to NICHD 2018 definition. [14]
Definition of abbreviations: IPPV=intermittent positive pressure ventilation; NIPPV=non-invasive positive pressure

A premature infant (<32 weeks' gestational age) with BPD has persistent parenchymal lung disease, radiographic confirmation of parenchymal lung disease, and at 36 weeks PMA requires 1 of the following FiO_2 ranges/oxygen levels/O_2 concentrations for ≥3 consecutive days to maintain arterial oxygen saturation in the 90%–95% range.					
Grades	**Invasive IPPV***	**N-CPAP, NIPPV, or nasal cannula ≥ 3 L/min**	**Nasal cannula flow of 1–<3 L/min**	**Hood O_2**	**Nasal cannula flow of <1 L/min**
I	—	21	22–29	22–29	22–70
II	21	22–29	≥30	≥30	>70
III	>21	≥30			
III(A)	Early death (between 14 days of postnatal age and 36 weeks) owing to persistent parenchymal lung disease and respiratory failure that cannot be attributable to other neonatal morbidities (eg, necrotizing enterocolitis, intraventricular hemorrhage, redirection of care, episodes of sepsis, etc).				

*Excluding infants ventilated for primary airway disease or central respiratory control conditions. Values are percents.

Jensen et al. in 2019 assessed infants <32 weeks' GA to determine which definition of BPD best predicts death or serious respiratory complications at 18-26 months of correct age. [15] They found that the respiratory support at 36 weeks' PMA, independently of oxygen therapy (current or past), best predicted these outcomes. At this timepoint the patients was classified as follow:

- No BPD: no respiratory support;
- grade 1: nasal cannula ≤ 2 L/min;
- grade 2: nasal cannula > 2 L/min or non invasive positive airway pressure;
- grade 3: invasive mechanical ventilation.

These criteria correctly predicted death or serious respiratory morbidity in 81% of cases.

Pathogenesis

The pathogenesis of BPD is complex and still not completely understood. To date, the new BPD is mainly considered a pulmonary development disorder, coupled with the potential damage of numerous risk factors for the immature lung. [1]

Pulmonary development of the fetus is divided into 3 periods: the embryonic period, the fetal period and the postnatal period (Figure 3).

The embryonic period goes from fertilization to the 7th gestational week. At this stage the lung precursor appears, at the end of the 4th week of gestation, from a ventral bud of the future esophagus. The epithelial component derives from the endoderm while the connective tissue from the mesoderm.

The fetal period is divided into:

- pseudoglandular stage (5-17 weeks): in this phase the lung looks like a tubule-acinar gland. It is characterized by continuous growth and branching of the periphery of the epithelial tubes. That leds to the formation of the conductive airways and the appearance of the acinar outliners. The arterial branches accompanying the airways are deposited during this phase;
- canalicular stage (17-26 weeks): the clusters grow by peripheral branches, by elongation of each tubular branch and by a marked enlargement of the distal air spaces. This new airways (approximately three branch orders) is called canaliculi. This process is accompanied by an increase in the capillaries, which begin to organize themselves around this air space, in close contact with the epithelial cells, forming the first air-blood barrier. In this stage there is the differentiation of the cuboidal epithelium into type I and type II pneumocytes and the start of the surfactant production.
- saccular stage (24 weeks-birth): at the beginning of this phase the airways terminate in clusters of thin-walled terminal saccules. These saccules produce the last generations of airways, some further prospective alveolar ducts and, at the outermost periphery, the alveolar sacs. There is the maturation of the surfactant system, the thinning of the connective tissue and the growth of the pulmonary parenchyma.

The postnatal period goes from birth to 1-3 years of life. In this stage there is the alveolarization phase, through a septation process that greatly increases the gas exchange surface area. In the human lung 90% of all alveoli are formed after birth. [16]

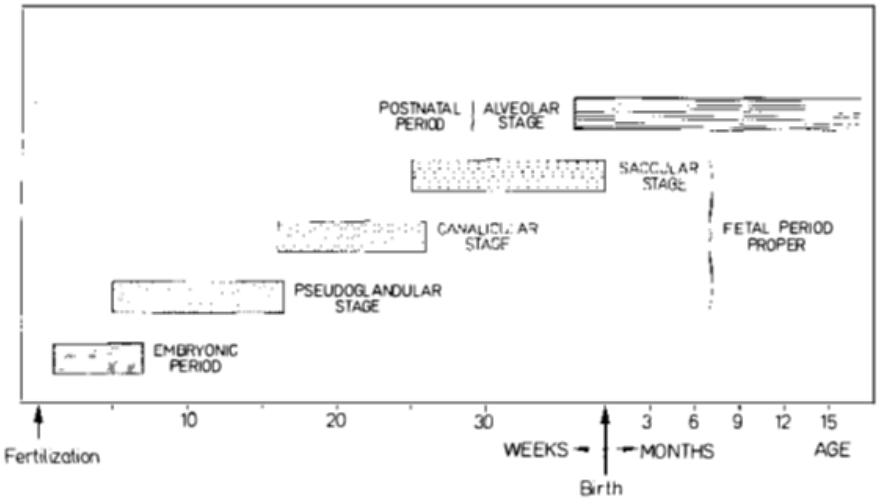

Figure 3. Fetal and postnatal lung development. [16]

As the survival of more immature newborns (23-26 weeks) increases in the last decades, many infants born in the canalicular or saccular lung development phases. At such an early stage of lung development, the impact of pathogenic noxae acting in the pre- and postnatal period leads to an arrest in normal lung development with an alteration of the normal alveolarization and capillarization processes, leading to a reduced number of alveoli and capillaries and a reduction in the total area available for gas exchange. In fact, lungs of infants with BPD are characterized by fewer, larger and simplified alveoli associated with dysmorphic vasculature. A key mechanism by which pathogenic noxae act in initiating and sustaining lung damage is inflammation [17]. Moreover the damage active the establishment of repair mechanisms in a vicious circle of continuous damage and repair that progressively worsens the lung structure. [11] From this it follows that the basic condition for the development of BPD is prematurity. Indeed, it is known that the incidence of BPD is inversely related to gestational age. [18] Genetic predisposition also plays an important role in the pathogenesis of BPD, as disease concordance was shown to be greater in monozygotic twins. [19] Genetic susceptibility probably is in relation to genes that regulate inflammation, surfactant expression, or vascular and lung development. [20]

Summarizing, BPD is a multifactorial condition caused by an injury on the immature lung in a complex interaction between genetics and exposure to prenatal and postnatal environmental factors, leading to a disruption in the normal lung development (Figure 4). [21]

The main risk factors (antenatal, at birth and postnatal) involved in the pathogenesis of BPD are reported below.

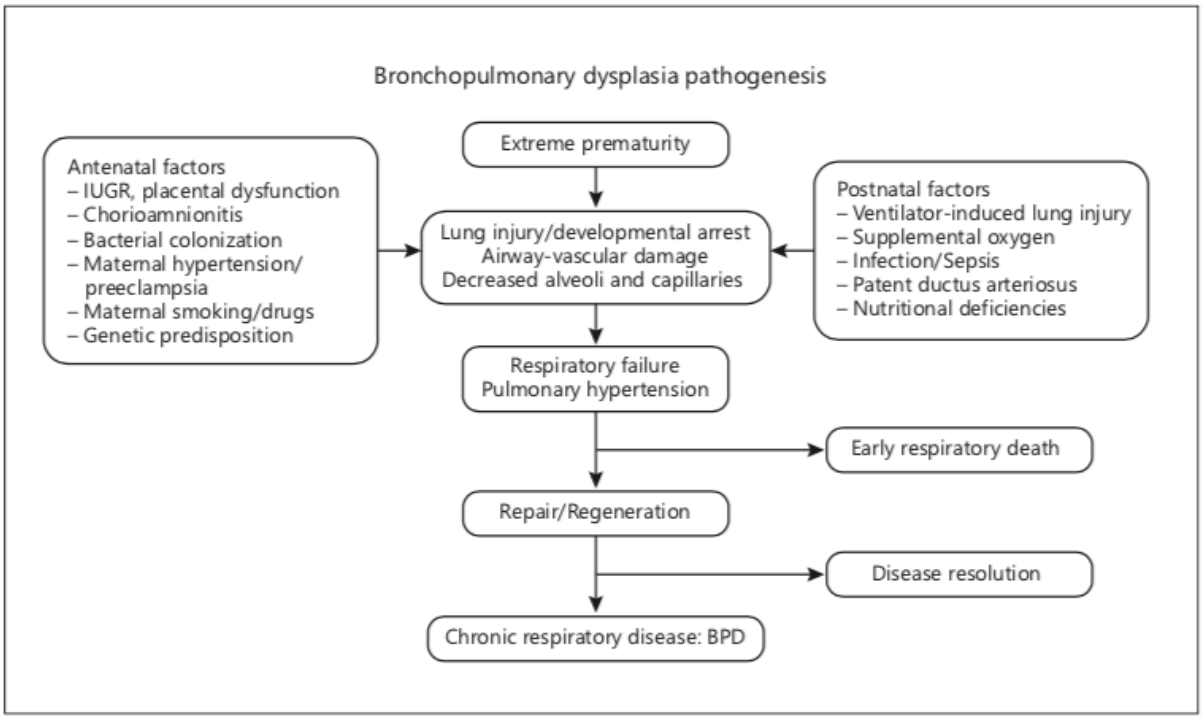

Figure 4. Algorithm for pathogenesis of BPD. [21]

Antenatal risk factors

Intrauterine growth restriction (IUGR): the presence of fetal growth restriction is an independent risk factor for the development of BPD. [22] it has been hypothesized that the mechanism that restricts growth during fetal life is also responsible for impaired growth and pulmonary maturation. In particular, an imbalance in proangiogenic and antiangiogenic cytokines, typical of women with preeclampsia and IUGR fetuses, would also be responsible for the reduced pulmonary maturation. Furthermore, chronic hypoxia, characteristic of the pathogenesis of IUGR, would interfere with lung development through an increase in TGFβ. [22]

Chorioamnionitis: chorioamnionitis is the inflammation of chorion and amnion, the membranes that surround the fetus. Although high values of IL-6, IL-8, IL-1β, tumor necrosis factor (TNF)-α in the amniotic fluid seemed to be associated with an higher risk of BPD, [23] [24] the role of chorioamnionitis as an independent risk factor for BPD is still debated. In a 25-year cohort study it was found that chorioamnionitis is associated with preterm labor and neonatal sepsis, however after adjusting for these two variables, BPD was not found to be associated with exposure to chorioamnionitis, [25] as previously described by Lahra et al. [26] Further studies are needed to settle the question.

Maternal hypertension and preeclampsia: as mentioned above, the imbalance of pro- and anti-angiogenic cytokines, typical of pregnancy-induced hypertensive disorders, interferes with normal lung development. However, the EPIPAGE 2 cohort study demonstrated that BPD is associated with hypertensive disorders when accompanied by intrauterine growth restriction but hypertension alone was not an independent risk factor for BPD. [27] Bose et al. also came to the same conclusion. [22]

Maternal smoking: intrauterine exposure to maternal smoking has been associated with the development of BPD with a OR od 2.21. [28] This observation has been confirmed from a Canadian and German study on VLBW infants. [29]

Genetic predisposition: BPD has a strong genetic predisposition, in fact greater concordance in the development of BPD was found between monozygotic twins compared to dizygotic twins. [19] Various genes have been candidates to be associated with the development of BPD, including genes linked to the expression of inflammatory cytokines such as IL-4, IL-10, TGFβ, TNFα and genes linked to surfactant proteins. [20] Carrera et al. performed a whole exome sequencing to identify non-common variants in patients with BPD. [30] The top candidate genes highlighted were nitric oxide synthase 2 (NOS2), matrix metalloproteinase 1 (MMP1), C-reactive protein (CRP), lipopolysaccharide-binding protein (LBP) and the toll-like receptor (TLR) family. [30] Also Li et al. performed a whole exome sequencing, identifying 258 genes rare nonsynonymous mutations in patients with BPD. [31] These genes were involved in processes for development of pulmonary structure and function including collagen fibril organization, morphogenesis of embryonic epithelium, and regulation of Wnt signaling pathway. [31]

Risk factors at birth

Prematurity and low birth weight: prematurity and low birth weight are the most important risk factor, with an incidence of BPD which is inversely related to both of them. [32] This is easily understood on the basis of the above reported pathophysiology.

Male sex: male infants have an higher risk of developing BPD as compared to females. [33]

Ethnicity: black race is associated with a lower risk of developing BPD and this advantage persists across all GAs, as demonstrated in a large cohort study in preterm infants <29 weeks' GA. [34]

Postnatal risk factors

Mechanical ventilation: ventilator-induced lung injury (VILI) are mainly determined by barotrauma, volutrauma, atelectrauma and biotrauma. [35] Barotrauma is the lung damage generated by excessively high pulmonary pressures while volutrauma is a lung injury caused by lung overdistension. Animal studies have shown that the major factor in VILI is volutrauma, not the elevated lung pressures per se which cause damage if they generate excess lung expansion. [36] Atelectrauma is the damage that occurs when ventilating with low lung recruitment causing repetitive collapse of the alveoli and airways, surfactant dysfunction and regional hypoxia. [36] Biotrauma is the damage induced by the inflammatory cascade. The mechanisms mentioned above (barotrauma and volutrauma) but also sepsis and oxygen toxicity are the main causes of biotrauma. [36] [37]

Hyperoxia and oxidative stress: oxidative stress plays an important role in the induction of lung damage and the development of BPD. [38] Oxidative stress derives from an imbalance between pro-oxidant and anti-oxidant factors. Premature infants are at high risk of oxidative stress due to the immaturity of antioxidant systems, increased susceptibility to infections and exposure to free iron. [38] Hyperoxia is one of the main factors that produce oxidative stress in preterm infants, increasing reactive oxygen species (ROS) and the expression of proinflammatory cytokines. [37] Oxygen radicals produce endothelial peroxidation, increased vascular permeability, interstitial, alveolar and airway edema resulting in lung injury. [38] At birth newborns are exposure to relative hyperoxia as the oxygen concentration in utero was lower. Additionally, most very low birth weight babies require higher concentrations of oxygen for resuscitation at birth. Furthermore, due to its physiological role, the lung is the organ that is most exposed to high concentrations of oxygen. [39] Many studies in vivo have demonstrated oxygen toxicity in animal models of mice, rabbit and baboons and oxygen toxicity-induced lesions in a premature lung were very similar to those present in BPD. [40] [41] [42] For all these factors, lungs of the premature infants are particularly exposed to oxygen damage and oxidative stress.

Postnatal systemic and pulmonary infections: postnatal sepsis is a known risk factor for BPD. [43] Lung infections and systemic sepsis induce lung damage by activating the inflammatory cascade and the release of inflammatory mediators and oxidative stress. [37]

Patent ductus arteriosus (PDA): the role of PDA as an independent risk factor for the development of BPD still remains controversial. The relationship between PDA and BPD would seem more of an association rather than a causal relationship. The pulmonary overflow caused by the left-right shunt

causes a reduction in lung compliance, [44] which would result in a greater need for invasive respiratory support and with higher oxygen concentrations, which in turn would lead to lung damage. [37] Recent randomized controlled trials on preventive PDA closure have not shown a reduction in the incidence of BPD in treated patients. [45] [46]

Microbioma: respiratory tract dysbiosis is recently thought to play a role in the development of BPD. In contrast with what was believed in the past, the respiratory tract of newborns is not sterile at birth. [47] Colonization with commensal bacteria begins in utero, [48] and respiratory tract microbiome is already present at birth, similar in extremely low birth weight infants and full term neonates independently from gestational age. [49] Furthermore, the microbiome undergoes an evolution with a progressive increase in bacterial loads and diversity. It has been hypothesized that prenatal and postnatal factors such as chorioamnionitis, antibiotic therapy, type of birth, type of enteral nutrition, sepsis can influence the microbiome, leading to dysbiosis of the respiratory tract, potentially activating the inflammatory process which can exacerbate BPD. [50] In infants with BPD it has been reported a progressive dysbiosis of the respiratory airways with a decreased bacterial diversity. [47] [49] Lohmann et al. described an increase in Firmicutes and a reduction in Proteobacteria and the progressive reduction in relative abundance of Acinetobacter sp. in BPD infants, with increasing amounts of Staphylococcus and Klebsiella. [47] On the contrary Vivek Lal et al. reported an increased phylum Proteobacteria and decreased phyla Firmicutes and Fusobacteria in infants with BPD. Lal et al. also showed that the early microbiome of infants who subsequently develop BPD was different with reduction in Lactobacillus. [49, 51] Moreover it is known that the colonization of respiratory airways with the genital mycoplasma species (Ureaplasma Parvum and U. Urealyticum) of preterm infants is a risk factor for the development of BPD, [52] supporting the role of the microbiome in the disease. However, the role of dysbiosis in the pathogenesis of BPD deserves further investigations.

Strategies for the prevention of BPD

Since BPD is a condition that develops over time from birth, also in relation to exposure to postnatal risk factors, and since currently there is no effective treatment available for the disease, the primary objective is to prevent its development.

Since prematurity is the underlying factor in the pathogenesis of BPD, all strategies aimed at reducing preterm delivery have the secondary effect of reducing BPD.

After birth, the main strategies for preventing the onset of BPD are listed below.

Oxygen supplementation: a Cochrane systematic review on adequate oxygen saturation targets for preterm infants showed a reduction in BPD risk with low targets (SpO_2 85-89%) compared to high targets (SpO_2 91-95%). [53] However, low targets are correlated with an increased risk of mortality and necrotizing enterocolitis. For that reasons it is recommended to use an oxygen saturation target between 90-95% in case of need for oxygen supplementation; [54]

Protective ventilatory strategies: over time, ventilation strategies have been progressively modified to ensure adequate gas exchange with the minimum level of ventilation parameters to avoid VILI. The combination of these strategies has been called "gentler ventilation". In particular, the use of volume-targeted ventilation strategies, [55] early extubation within 7 days of life and avoiding reintubation have been correlated with a reduction in the incidence of BPD; [56]

Non-invasive respiratory support: systematic review and meta-analysis support early initiation of nasal continuous positive airway pressure (CPAP) in the delivery room for infants at risk for BPD. [57] Other non-invasive ventilatory strategies, such as nasal intermittent positive pressure ventilation, have not been shown to reduce the risk of BPD compared with CPAP; [58]

Exogenous surfactant with budesonide: administration of exogenous surfactant alone is not associated with a reduction in the risk of BPD. [59] However intratracheal administration of surfactant with budesonide reduce the incidence of BPD or death; [60] among the methods for administering surfactant, less invasive surfactant administration (LISA) would seem the one with the lowest incidence of BPD in premature infants; [61]

Caffeine: caffeine therapy is associated with a reduction in the incidence of BPD; [62]

Postnatal corticosteroids: the use of postnatal corticosteroids has been controversial for a long time since, despite the beneficial effects on lung function, the potential adverse effects, on neurological development in particular, have limited their use. However a recent systematic review and network meta-analysis has concluded that a short course (<8 days) of cumulative dose of 2-4 mg/kg in the second week of life of systemic dexamethasone might be the most appropriate regimen of postnatal corticosteroids for preventing the risk of BPD or mortality at 36 weeks' PMA with reasonable safety; [63]

Vitamin A: supplementation with vitamin A reduces the risk of BPD in ELBW infants; [64]

Nutrition and fluid management: a relative fluid restriction, early introduction of enteral feeding and optimization of energy intake in parenteral nutrition are recommended, since insufficient energy and excessive fluid intakes and low intake of enteral feeding in the first week of life are associated with

an increased risk of BPD. [65] Furthermore, the administration of human milk (donor milk or fresh expressed maternal milk) is associated with a reduction in BPD; [66] [67]

Antenatal corticosteroids: unlike neonatal mortality and major neonatal morbidity, antenatal corticosteroids do not reduce the risk of developing BPD. [68]

In addition, innovative therapies for preventing BPD include insulin-like growth factor 1 (IGF-1) and mesenchymal stem/stromal cells (MSCs) and their effectors, extracellular vesicles (EV-MSCs), both still in an experimental phase. [17]

Outcomes

Prematurity is increasingly considered a chronic condition as the disruption of the normal development of organs and systems can lead to consequences that last into adulthood. [69] Among these, also BPD is associated with long-term complications, in particular neurological and respiratory outcomes. [70]

Respiratory outcomes. Infants with BPD are more likely to develop lower respiratory tract infections, particularly viral infections such as respiratory syncytial virus (RSV) in the first 2 years of life. These pulmonary exacerbations lead to an increase in re-hospitalizations rate, although re-hospitalizations are not different between premature infants with and without BPD. [71] [72] During childhood and adolescence, these patients are more prone to develop asthma-like respiratory symptoms with coughing and recurrent wheezing associated with some degree of bronchial hyperreactivity. However, unlike asthma, bronchial hyperreactivity in these patients is characterized by less reversibility, less response to inhaled steroids and less exacerbation of symptoms. This suggests that the mechanisms underlying bronchial hyperactivity are different from actual asthma. [71] [1]

BPD can also be complicated by pulmonary arterial hypertension, with a prevalence between 17% and 43%, which in turn is complicated by increased mortality and morbidity. [73]

Longitudinal studies have shown that premature infants, particularly those with BPD, have some degree of impairment of lung function with airflow obstruction which persists into childhood and adolescence. [71] [73] [74] [75] In particular, the mean difference for $\%FEV_1$ in those born preterm with BPD, diagnosed as O_2 dependence at 28 days of life and 36 weeks' PMA, was 16.2% and 18.9%, respectively. [76] Some studies also describe a more rapid decline in lung function over time in preterm survivors, particularly with BPD. [77] However, this is still debated as other studies show impaired lung function but without a more rapid decline. [74] [78] In any case, these patients enter

adulthood with impaired lung function, not reaching their potential peak pf pulmonary function usually achieved at 20-25 years of age, therefore being at high risk of developing chronic obstructive pulmonary disease (COPD) later in life. [73] [79]

Neurological outcomes. Children with BPD are more prone to develop neurological problems. BPD is significantly associated with cerebral palsy. [80] Other neurological disorders reported in BPD survivors are: specific movement disorders, poorer fine and gross motor skills, visual and auditory problems, cognitive and motor delay, lower IQ,, attentional impairments, increased rate of attention deficit-hyperactivity disorders (ADHD), speech and language disorders, memory difficulties, visual-spatial perceptual deficits, executive deficits, more school-based problems which more likely require educational assistance, increased behavioral problems. [70]

Biomarker based approaches

Since BPD is a disease that develops over time, early recognition of infants at high risk or recognition of BPD at an early stage is critical for implementing preventive strategies and minimizing exposure to postnatal risk factors in these patients. With this in mind, numerous biomarkers capable of predicting the development of BPD have been considered. These biomarkers have been studied in different biological fluids: maternal blood, cord blood, peripheral blood, urine, tracheal aspirate. Since the main pathogenetic mechanism of BPD is damage caused by inflammation and impaired lung development, the research of biomarkers has focused on inflammatory mediators and growth factors involved in the process of alveolarization and vascular development. The biomarkers that have shown consistent results are: IL-6, -8, -10, MCP-1, CC10, TIMP 2, soluble E-selectin, KGF, TGFb1, Angiopoietin 2 and IFNγ, VEGF. [81]

Table 4 shows a summary of humoral biomarkers of BPD reported in literature.

Table 4. Summary of humoral BPD biomarkers reported in literature.

	Biomarkers in BPD	
	increased	*decreased*
MATERNAL BLOOD	AFP and/or HCG [81]	unconjugated estriol [81]
CORD BLOOD	Endostatin [54] VEGF [54] PDGF-BB [54] BMP-10 [54] FGF-19 [54] HGF [54]	Ang1 [54] PIGF [54]
PERIPHERAL BLOOD	Ang 2 [54] Nitrites [54]	L-arginine [54] Ang1 [54]

	IL-1β [54] IL-6 [54] IL-8 [54] E-selectin [54] IFNγ [54] GCSF [54] miR-219 [54]	FGF-18 [54] PDGF-AA [54] IL-17 [54] RANTES [54] TNF-β [54] soluble L-selectin [54] MCP-1 [54] TGFβ [54] KL-6 [54] MMP/TIMP-1 [54]
TRACHEAL ASPIRATE	VEGF receptor [54] IL-6 [54] IL-8 [54] NF-κB [54] MCP-1 [54] MCP-2 [54] MCP-3 [54] IL-1β:ILRA ratio [54] MMP-8 [54] MMP-9/TIMP-1 [54] TGFβ [54] Elastase [54] myeloperoxidase [54] xanthine oxidase [54] catalase [54] total sulfhydryls [54] epithelial lining carbonyls [54] 3-chlorotyrosine [54] malondialdehyde [54] miR-34a [54]	VEGF [54] TIMP-2 [54] MMP-2 [54] NGAL [54] miR-876-3p [54] miR-378b [54] miR-20a-50 [54] +miR-20b-5p [54] miR-1254 [54] miR-1252-5p [54]
URINE	8-OHdG [81] Bombesin-like peptide [81]	

Unfortunately none of these have been validated and none of these are of routine use in clinical practice. There are many limitations that prevent theirs use. Most of these biomarkers, taken alone, have little predictive value and do not add much more to the predictive capacity of clinical variables such as gestational age, birth weight, degree of respiratory disease etc. [82] Furthermore, it has been reported that a profile of biomarkers, rather than a single biomarker, and its variation over time has a greater predictive power than the single concentration value of just one biomarker. [82] It must also be considered that specific analytical methods, present only in research laboratories, are required for the analysis of many of these biomarkers. [82] Finally, the predictive capacity is also limited by the fact that many of these biomarkers are not lung-specific (except those in tracheal aspirate and bronchoalveolar lavage) and are influenced by potential inflammatory states in other parts of the body. [83]

In this context, in recent years, the most modern technologies have been applied in order not only to identify a specific profile of the disease but also to investigate its pathogenetic mechanism more in depth. The new technologies, the so-called “omics” sciences to which genomics, transcriptomics,

proteomics, microbiomics and metabolomics belong, study a biological system as a whole, thus including the dynamic interactions of its components and how these are modified by the introduction of a change. [84] Specifically, the reciprocal interactions between genome, RNA, proteins, metabolites are evaluated. [84] In the "omics" sciences the entire set of molecules (proteins, RNA, DNA, etc.) of a given system are described and this large data sets are analyzed through bioinformatics methods. In this way, a specific profile is obtained, which is the response of a system to a modification. The interesting thing is that these approaches often do not foresee "a priori" hypotheses, on the contrary the opposite mechanism is used: from the specific profile obtained of a given condition, it is possible to hypothesize the pathogenetic mechanism. Furthermore, this specific signature in at-risk individuals can be used to predict the specific condition, in this case the development of the disease. [85]

Both genomics, proteomics, microbiomics and metabolomics have recently been applied to the study of BPD.

In Table 5 there is a summary of the main results of the application of proteomics, genomics and microbiomics. [85]

Table 5. Summary of BPD biomarkers resulted from the application of proteomics, genomics and microbiomics reported in literature.

		Biomarkers in BPD	
		increased	*decreased*
PROTEOMICS	*Tracheal aspirate*	surfactant protein-A2 [54] annexin-3 [54] calcium and integrin binding protein-1 [54]	leukocyte elastase inhibitor [54] calcyphosine [54]
MICROBIOMICS	*Tracheal aspirate*	Enterobacteriae [54] Ureaplasma [54] Staphylococcus [54]	Lactobacillus [54]
GENOMICS	*Blood*	inflammatory response genes [54] CD44 [54] phosphorus oxygen lyase activity [54] connective tissue mast cells [54]	T cell receptor related activation genes [54]

Metabolomics

Among the "omics" sciences, metabolomics is the most recent. Metabolomics allows the identification and quantification of all metabolites present in a complex biological system. Metabolites are building blocks of cellular function and can be peptides, lipids, organic acids,

vitamins, minerals, drugs, amino acids, nucleic acids, carbohydrates, fatty acids, hormones, drugs and any other small molecules <1000 Da. The set of metabolites is not static but is continuously modified by the reciprocal interactions between the genome and environmental factors such as nutritional factors, age, microbiota, drug or toxic exposure. Therefore, metabolomics allows to identify changes in the composition of metabolites in relation to exposure to these factors. For this reason it is considered the "omics" science that comes closest to describing the phenotypic expression of a biological system, thus allowing to analyze the genotype-phenotype relationship. [84] [85]

There are two approaches that can be applied to metabolomics: target and untargeted. The target approach consists in identifying a limited number of metabolites already known and it is usually used for verifying hypothesis. [84] [86] On the contrary, the untargeted approach analyzes the entire data set of metabolites of a biological system, identifying known metabolites and also those not yet identified, as well as highlighting known and even unknown and unexpected changes in response to an event. The advantage of this approach is that data are collected without pre-existing knowledge and without a priori theories, thus allowing to generate new hypotheses on the basis of the objective data that emerge from the analysis. In fact, this type of approach is generally used in hypothesis-generation studies. [84] [86] The metabolite fingerprinting is an untargeted approach that aims at identifying a set of metabolites that allow to discriminate 2 different groups, without necessarily identifying all metabolites present in the system. Applying it to clinical practice, this technique allows to identify a profile of biomarkers able to distinguish between healthy patients and patients affected by a given disease and also to distinguish subgroups of different phenotypes within the same disease. [84]

An advantage of metabolomics is that the analyzes can be performed in different biological fluids such as blood, urine, tracheal aspirate, bronchoalveolar lavage, etc. and that the amount required for the analyzes is very small, from less than 20 μL to 300 μL. [84]

The main platforms for performing metabolomic analyzes are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). [84] ^{1}H-NMR spectroscopy allows the detection of molecules containing protons and different metabolites generating different signals in the NMR spectrum. NMR spectroscopy identifies the molecular structure of metabolites, both single and in complex mixtures. The advantages of NMR spectroscopy are: the speed of execution, the non-selectivity and that it does not require sample preparation. [84] MS allows the identification of metabolites by generating a spectrum in which metabolites rank based on their mass. Before MS, it is necessary to perform the separation of the sample components using chromatography. An

advantage of MS is the greater sensitivity over NMR spectroscopy. Recent applications of MS in metabolomics are based on instruments like Quadrupole Time-of-Flight (Q-ToF). [84]

The huge amount of data generated by NMR spectroscopy and MS can only be managed with bioinformatic methods capable of extracting the data with appropriate multivariate statistical approaches, called "pattern recognition" method. The pattern recognition method can be unsupervised and supervised. The unsupervised method, such as principal component analysis (PCA), transforms the data obtained with analytical techniques into plots, thus making them visually understandable and easy to interpret. This method is very useful for identifying whether there are clusters of different metabolites between two groups. The supervised methods, such as partial least squares-discriminant analysis (PLS-DA), use a training set of samples (of known classification) to create a mathematical model that is then used to test an independent dataset.

After these steps, a key point is the structural identification of the metabolites involved. [84] Online databases are of fundamental importance for the molecular identification of metabolites, of which the most important is the Human Metabolomic Database (HMDB). HMDB is a freely available electronic database containing detailed information about small molecule metabolites found in the human body and it contains or links three kinds of data: chemical data, clinical data and molecular biology/biochemistry data. To date, the HMDB contains 220,945 metabolites. [87]

Metabolomics applied to bronchopulmonary dysplasia: what is already known

Metabolomics is a promising field of research and several studies for identifying early markers of BPD development are reported in literature.

Fanos et al. studied urinary metabolomic at birth in 36 newborns <29 weeks' GA with birth weight <1500 g, of that 18 were enrolled as cases (infants who subsequently developed BPD) and 18 as controls (infants without BPD). [88] They analyzed samples with ^{1}H-NMR spectroscopy and PLS-DA model. The results showed that a different metabolomic profile was already present in urine at birth in infants who subsequently developed BPD compared to controls. In particular in the BPD group they found the presence of lactate, taurine and trimethylamine- N-oxide (TMAO) (not present in the control group), increased levels of myoinositol and reduced levels of gluconate. [88]

Also Pintus et al. in 2019 performed a study on urinary metabolomics. They collected urine samples at DOL 7. Eighteen newborns <32 weeks' GA and with birth weight <1500 g were enrolled, including 11 controls (without BPD) and 7 cases (neonates who subsequently developed BPD). [89] Samples were analyzed with ^{1}H-NMR spectroscopy associated with multivariate statistical analysis. They

found the presence of a markedly different metabolomic profile in infants who subsequently developed BPD compared to controls. The discriminating metabolites between the two groups were: alanine and betaine (increased in the BPD group) and TMAO, lactate and glycine (reduced in the BPD group). [89] Lactate in BPD patients, in these two urinary metabolomics studies, was found to be reduced at birth and increased at DOL 7. The increase in lactate at birth is an expression of the activation of anaerobic glycolysis due to hypoxia. Its reduction to DOL 7 has been interpreted as a compensatory mechanism: lactate can be used as a substrate for gluconeogenesis and therefore its reduction can be an expression of oxidative stress. [89] TMAO plays a role in osmoregulation and, like lactate, it was found to be increased at birth and reduced at DOL 7. The formation of TMAO depends on the intestinal microbiota, therefore its reduction from birth suggests a modification of the intestinal microbiota. [89] Taurine has a role in osmoregulation, membrane stabilization, renal cell cycle and apoptosis and calcium homeostasis. Taurine is released from the tissues as a protective mechanism against hypoxia and to maintain osmotic balance. [88] It was supposed for that reason that it was found to be present in BPD patients. Under conditions of anaerobic metabolism, pyruvate cannot enter the Krebs cycle and it is transformed into alanine and lactic acid. Therefore, the increase in alanine is an expression of the increase in anaerobic metabolism in response to hypoxia. [89] Betaine is a donor of methyl groups and is involved in DNA methylation processes. Oxidative stress, a well-known pathophysiological mechanism of BPD, has an important impact on DNA control and signal transduction mechanisms. [89] Glycine has also been found to be reduced in BPD patients. Glycine plays a role in the synthesis of glutathione, which is a powerful antioxidant of free radicals and other reactive oxygen species. Therefore, in conditions of oxidative stress, glutathione is consumed and consequently the glycine levels are reduced. [89] [90]

Baraldi et al. performed a metabolomic analysis on amniotic fluid obtained from amniocentesis between 21 and 28 gestational weeks. Twenty-one samples of preterm infants (<37 weeks' GA) were analyzed, of which 10 with BPD and 11 without BPD. They found in the BPD group higher levels of leucinic acid, hydroxy fatty acids (4-Hydroxy- 3-methylbenzoic acid and 2-hydroxy caprylic acid), oxy fatty acids (3-oxo-dodecanoic acid) and a metabolite ascribable to a sulphated steroid. [90] The group without BPD was characterized by higher levels of S-adenosyl-methionine and aminoacid chains and 3β,16α-Dihydroxyandrostenone sulfate (a metabolite ascribable to DHEAS). [90] Also S-adenosyl-methionine has a role in the glutathione synthesis, in particular S-adenosyl-methionine is a precursor, so its reduction is an expression of increased need of glutathione caused by an oxidative stress

environment. Moreover the increase in 3β,16α-Dihydroxyandrostenone sulfate in no-BPD group confirm the association between BPD and adrenocortical insufficiency. [85]

All these studies together confirmed the key role of oxidative stress in the pathogenesis of BPD.

Piersigilli et al. in 2019 performed a metabolomic study on tracheal aspirate (TA) in the first week of life in neonates <30 weeks' GA in mechanical ventilation. They enrolled 68 neonates, of which 44 with BPD and 24 without BPD and they collected 160 TA samples. Samples were analyzed with MS coupled with PLS-DA methods. [83] They found a cluster of 53 metabolites characteristic of BPD, of which 18 highly significant. The most significant metabolites were histidine, glutamic acid, citrulline, glycine, isoleucine, asparagine, serine, taurine, spermidine, acylcarnitines C16-OH and C18:1-OH, which all presented higher level in the BPD group. [83] Among these, a metabolite of particular interest is citrulline, which levels are increased in patients with BPD. Citrulline is a precursor of arginine, which in turn is a substrate for the formation of nitric oxide. Nitric oxide plays a role in the prevention of pulmonary hypertension which may be associated with BPD. Therefore, the increase in citrulline has been interpreted as an attempt to increase nitric oxide levels in BPD patients to protect against the development of pulmonary hypertension. [83]

Carraro et al. analyzed 20 adolescents BPD survivors compared with 15 healthy adolescent controls. They investigated their exhaled breath condensate (EBC) applying the metabolomic approach using MS coupled with multivariate statistical analysis. [91] They found an altered complex lipid profile in patients BPD survivors: lyso-phosphatidylcholine, platelet activating factor, unsaturated phosphatidylcholines, plasmenyl-phosphatidylserine. [91] For the control group, the characteristic metabolites were hydroxyeicosapentaenoic acid and phosphatidylserine. Authors concluded that alteration of these metabolites in BPD survivors could indicate that an altered surfactant composition, lasting beyond infancy, has a possible role in the pathogenesis of BPD. [91] [92]

A role of dyslipidemia in the development of pulmonary hypertension associated to BPD has also been proposed by the study of La Frano et al. They performed both untargeted and target metabolomic analysis on umbilical cord blood plasma of 42 preterm infants (<37 weeks' GA): 21 cases with BPD-associated PH and 21 controls with BPD without PH. [92] They also perform an independent validation study on 20 newborns matched for GA and BPD status (moderate-severe), 10 with BPD-PH and 10 BPD-without PH. [92] They found dyslipidemia in infants who subsequently develop BPD-associated PH, characterized by a reduction in choline-containing phospholipids (phosphocholine and sphingomyelin) and elevation in choline. This result suggests an immaturity of lipid metabolisms and a role of dyslipidemia. [92]

Lal et al. studied the relationship between airway microbiome and metabolome at birth. They collected TA samples within 6 hours of life and before surfactant administration of 30 neonates (15 BPD-predisposed and 15 BPD-resistant) <29 weeks' GA. [93] They found an increase in Proteobacteria abundance and a reduction in Lactobacilli in BPD-predisposed neonates, a reduction in acetyl-CoA/propionyl-CoA carboxylase ortholog gene and genomic pathways of caffeine metabolism in the microbiome of BPD-predisposed infants. [93] The airway metabolic profile of these infants were also characterized by decreased metabolites of fatty acid activation and androgen and estrogen synthesis pathways. [93] The changes in the lipid metabolism in airway microbial metagenome and metabolome and in airway metabolome at birth of the BPD-predisposed infants suggest that metabolic activity of the microbiome may affect the metabolome. [93]

Lewis et al. in 2019 performed the first study of pharmacometabolomics applied to preterm infants with BPD. Pharmacometabolomics is a branch of metabolomics that studies the variation of the metabolome before and after a treatment, thus identifying subjects with a metabolome that predicts drug-response. Hypothetically it is useful in the individualization of therapies, to administer treatments only to those who would benefit from it, avoiding exposing non-responders to side effects. In this study, 10 preterm infants born before 32 weeks' GA treated with systemic dexamethasone were included. [51] Blood and urine samples collected 24 hours before treatment and 3-6 days after it were analyzed with untargeted metabolomics. They found 11 metabolites in serum and 15 metabolites in urine which were significantly different between pre and post treatment: gluconic acid in serum and isohexanoic acid in urine shown the largest decreased while serum caprylic acid and urine sucrose displayed the largest increase. [51] Furthermore, baseline xylitol levels in serum and saccharic acid in urine are the most associated with clinical steroid response. Urine gluconic acid lactone, uridine and mannitol were correlated with the degree of clinical dexamethasone response. All 3 metabolites (gluconic acid, uridine and mannitol) are implicated with the inflammation cascade and oxidative stress, known pathogenetic mechanisms of BPD. [51]

In Table 6 are summarized the principals metabolites involved in BPD, reported in literature.

Table 6. Summary of BPD biomarkers resulted from the application of metabolomics reported in literature.

	BPD		**No-BPD**
	increased	*decreased*	*increased*
Amniotic fluid	leucinic acid [90] hydroxy fatty acids (4-Hydroxy- 3-methylbenzoic acid and 2-hydroxy caprylic acid) [90]		S-Adenosylmethionine [90] 3β,16α-Dihydroxyandrostenone sulfate (DHEAS) [90]

	oxy fatty acids (3-oxo- dodecanoic acid) [90]		
Urine	Lactate at birth (present only in BPD) [88] Taurine at birth (present only in BPD) [88] TMAO at birth (present only in BPD) [88] Myoinositol at birth [88] Alanine at DOL 7 [89] Betaine at DOL 7 [89]	Gluconate at birth [88] TMAO at DOL 7 [89] Lactate at DOL 7 [89] Glycine at DOL 7 [89]	
Tracheal aspirate	Histidine [83] glutamic acid [83] citrulline [83] glycine [83] isoleucine [83] asparagine [83] serine [83] taurine [83] spermidine [83] acylcarnitines C16-OH and C18:1-OH [83] Proteobacteria [93]	Lactobacilli [93] acetyl-CoA/propionyl-CoA carboxylase ortholog gene [93] genomic pathways of caffeine metabolism [93] fatty acid [93] metabolites of androgen and estrogen synthesis pathways [93]	
Exhaled breath condensate	lyso-phosphatidylcholine [91] platelet activating factor [91] unsaturated phosphatidylcholines [91] plasmenyl-phosphatidylserine [91]		hydroxyeicosapentaenoic acid [91] phosphatidylserine [91]

AIM OF THE STUDY

The aim of the study is to apply the metabolomic approach in order to establish whether a biomarker profile exists at birth, capable of predicting the development of bronchopulmonary dysplasia in preterm infants less than 32 weeks gestational age.

The secondary aim is to characterized the urinary metabolome of BPD patients at birth in order to investigate whether altered metabolic pathways are still identifiable in the pathogenesis of BPD.

MATERIALS AND METHODS

Study design

This is a monocentric prospective observational case-control study.

Setting

Patient enrollment was conducted at the Neonatal Intensive Care Unit (NICU), Department of Women's and Children's Health, Hospital-University of Padova, Padova, Italy. It's a tertiary-level Center with 30 bed capacity, of which 15 intensive care and 15 sub-intensive care beds. It's a referral NICU for the eastern Veneto area, that covers about 3 million people. In 2019, 423 newborns were admitted, of that 80 (19%) were outborn. Among all admitted patients, 97 were very low birth weight infants (birth weight <1500 g) and 60 extremely low birth weight infants (birth weight <1000 g). In 2019, BPD was diagnosed in 30% of infants with birth weight <1500 g.

Population

Infants were enrolled on the basis of the following criteria.

Inclusion criteria:

- admission to the NICU;
- born at less than 32 weeks' gestational age;
- written informed consent for participation of a legally acceptable representative.

Exclusion criteria:

- major congenital abnormality or chromosomal abnormality;
- known or suspected congenital metabolic disease;
- refusal of consent.

Patients were enrolled from March 2015 until June 2020.

Patients with BPD were enrolled as cases, patients without BPD were enrolled as controls. Furthermore, cases have been classified according to the severity of BPD in mild, moderate and severe. Diagnosis and severity of BPD were defined using the National Institutes of Health consensus workshop definition of BPD. [11] BPD was diagnosed if the need for oxygen supplementation was present for at least 28 days. Severity of BPD was classified on the basis of respiratory support needed at 36 weeks' PMA or discharge (whichever comes first) for infants born at <32 weeks GA: "mild" if

breathing room air, “moderate” if need for < 30% oxygen and “severe” if need for ≥ 30% oxygen and/or positive pressure.

Sample collection

For each patient enrolled, urine sample of 2 ml was collected within 48 hours of life (T_0) using a non-invasive way (a cotton swab) and subsequently transferred into a tube. Samples were stored in a freezer at -80°C until metabolomic analysis.

Data collection

For each enrolled patient, clinical data was extracted from medical records. Seven clinical data that could potentially influence the development of BPD were recorded. Specifically: sex, gestational age (GA), birth weight, intra uterine growth restriction (IUGR), premature rupture of membranes (PROM), early onset of sepsis (EOS) and antenatal steroid treatment.

Personal information about potential and enrolled participants was collected, shared and maintained in order to protect confidentiality before, during, and after the study in a personal password-protected PC.

Metabolomic analysis

The analysis was performed at the Mass Spectrometry and Metabolomics Laboratory of the Women’s and Children’s Health Department, University of Padova, Padova, Italy. Untargeted metabolic profiling was performed on urine samples.

Urine samples were slowly thawed overnight at +4°C and then transferred to ambient temperature for the preparation. Each sample was stirred and centrifuged at 3600 g for 10 min at 10°C, then 15 µl of the supernatant from each sample were pipetted in a well of 384 wells plate, adding 240 µL of 0.1% formic acid (FA) solution (finale volume 225µL, dilution 1:15). All the procedures for the preparation were automatically managed by a robotic liquid handling system, Multiprobe II Ex (Perkin Elmer).

Untargeted metabolic profiling of urine samples was performed in positive electrospray ionization (ESI) mode on an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, U.K.) coupled to a Quadrupole Time-of-Flight (QToF) Synapt XS HDMS mass spectrometer (Waters MS Technologies, Ltd., Manchester, U.K.).

The mass range scan was of 20 to 1200 amu, in MS scan mode. The capillary voltage was set at 0.7KV; and the sampling cone voltage at 40 V. The desolvation gas flow was set at 800 L/h with temperature kept at 400°C. The cone gas flow was set at 20 L/h with temperature kept at 110°C. To correct for changes in environmental or experimental condition over the course of the analysis, Leucine-Enkephalin solution at a concentration of 100 pg/ml was injected periodically (every 30 s) as internal reference (i.e. lock mass).

For LC-MS analysis a Waters Acquity UPLC HSS T3 column 2.1 mm wide and 100 mm long packed with 1.8 µm beads was used and its temperature was kept at 50°C. The mobile phase flow rate was set at 0.5 ml/min. The gradient mobile phase consisted of water with 0.1% FA (A) and methanol with acetonitrile in a 90:10 ratio with 0.1% FA (B). Each sample run lasted 12 minutes and consisted of an isocratic phase of 5% B for 1 minute, a linear increase to 30% B in 2.5 minutes, a linear increase to 95% B in 3 minutes, an isocratic phase of 95% B for 1.5 minutes, a washout phase of 5% B for 3 minutes. For each run, 3 µl of sample were injected.

Quality Control samples (QC) and Standards Solution Samples (Mix) were used to assess reproducibility and accuracy during the analysis, and examine the metabolite content of the samples. The QCs were prepared from an aliquot (10µL) of each sample, pooled together and diluted with six different dilution factors (1:3, 5, 10, 20, 50, 100) with 0.1% FA solution in water, treated as the samples. The Mix consisted of nine compounds of known exact mass and retention time. The QCs and Mixes were injected at regular intervals of 15 samples during the sequence, together with blank samples, to identify specific ions from the mobile phase, and any contaminants.

The sequence for the analysis were prebuilt in Excel to randomize the samples injections and prevent any spurious classification deriving from the position of the sample in the sequence.

Data pre-processing

Raw data were extracted using Progenesis QI software (Waters Corporation, U.S.A.). The parameters were optimized through the preliminary processing of the QCs. Specifically, 0.5 was set as filter to import the raw data, and the QC in the middle of the sequence was selected as reference for the automatic retention time alignment of the samples in the sequence. The sensitivity of the automatic algorithm for peak picking was set at 5 in the time range from 0.4 to 8.0 min. As a result, the so-called time_mass variables (where “time” is the retention time and “mass” is the mass to charge ratio m/z of the spectral feature) were generated.

Features with at least one missing data in the QCs and more than 10% of missing data in the samples were eliminated. For each variable passing such a filter, missing data were imputed with a random number between zero and the minimum value measured for that variable. Data were calibrated on the basis of the local linear regression models obtained considering the trend of the QCs with the run order. Probabilistic quotient normalization was applied to take into account dilution effects. Variables with a coefficient of variation greater than 30% in the QCs were excluded.

Statistical analysis

Exploratory data analysis was performed by Principal Component Analysis (PCA) whereas Random Forest (RF) and PLS-techniques were applied to solve classification problems, where BPD and noBPD subjects were compared.

PCA [94] is an unsupervised multivariate technique able to summarize the data variation of a data table using a small set of score components, called principal components, calculated by linear combination of the measured features. The model of the data table obtained by PCA approximates the original data structure providing a less complex da-ta-representation, where the similarity between observations and the correlation structure among the measured features are preserved.

Random Forest (RF) [95] is a learning method for classification based on generating a large number of decision trees, each constructed using a different subset of the training set. These subsets are selected by sampling at random and with replacement (bootstrap) from both the observations and the predictors. The decision trees are then used to identify a classification consensus by selecting the most common output. Using Multi-Dimensional Scaling (MDS),it is possible to transform the proximity matrix obtained considering when two observations occupy the same terminal node in the forest, into a scatter plot that provides a low-dimensional representation of the observations.

PLS for classification (PLS2C) has been recently introduced to solve the general G-class problem [96], when correlation, redundancy and noise affect the data. It overcomes the weakness of the theoretical basis of PLS-DA and is suitable for multivariate investigations. Specifically, score components capable to separate the observations of the classes under investigation are built by linear combination of the measured variables. As a result, the relationship of similarity between the observations can be investigated exploring the score space of the model. Relevant variables can be highlighted by a procedure called “stability selection”. The idea underlying stability selection is that real structure in the data should be present consistently, and therefore should be found even under perturbation of the data by sub-sampling. Bootstrap is applied to extract N sub-samples from the

training set and, thus, PLS2C with variable selection based on variable influence on projection (VIP) applied to each sub-sample to obtain N classification models, each one based on a different optimal subset of variables. For each variable, the number of models whose optimal subset includes that variable is considered to calculate the so called "relevance score" that is used to select the relevant variables. The optimal subset is determined on the basis of the maximum Matthews Correlation Coefficient (MCC) calculated by 5-fold cross-validation. PLS2C can be constrained analogously to PLS2 [97] to force the score space to be orthogonal to some forbidden subspace.

Data analysis was performed by in house R-function implemented, using the R 4.0.4 platform (R Foundation for Statistical Computing, Vienna, Austria).

Matching BPD and noBPD neonates

The procedure implemented to extract the two groups of neonates, BPD and noBPD, from the eligible neonates is described in the following. The distance matrix between the neonates of the BPD group and the eligible noBPD neonates was calculated considering the clinical data recorded at birth. Given the neonate i and the neonate j, the distance between them was defined as

$$d(i,j)=\sum_{k=1}^{P} d_k(i,j)$$

where *P* is the number of clinical data. In the case of categorical data, the contribute was

$d_k(i,j)=0$ if the levels of the descriptor *k* were the same for the two neonates

or

$d_k(i,j)=1$ if the levels were different

or, in the case of numerical data, the contribute was

$d_k(i,j)=\left|x_{ki}-x_{kj}\right|/s$ if $\left|x_{ki}-x_{kj}\right|<s$

or

$d_k(i,j)=1$ if $\left|x_{ki}-x_{kj}\right|\geq s$

being x_{ki} the value of the descriptor *k* for neonate *i* and $s=\left[\max(x_k)-\min(x_k)\right]/n$. The parameter *n* was set equal to 5.

For each BPD neonate, the noBPD neonate with the minimum distance was selected and the pairs case-control obtained were sorted on the basis of the increasing distance. The pairs with the greatest

distance were iteratively excluded until the set of the selected BPD neonates and the set of the selected noBPD showed p-values of the t-test or of the Mann-Whitney test greater than α in the case of numerical data normally or non-normally distributed, respectively, and p-value of the Fisher's exact test greater than α in the case of categorical data.

Variable annotation

The relevant variables selected by data analysis were annotated by searching our in-house database of commercial standards, the METLIN metabolite database and the Human Metabolome Database using a different level of confidence. [98]

Four levels of metabolite identifications can be found:

1. Identified compounds;

2. Putatively annotated compounds (e.g. without chemical reference standards, based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries);

3. Putatively characterized compound classes (e.g. based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class);

4. Unknown compounds. [98]

Ethics Committee approval

The study was approved by the Ethics Committee of Padua Hospital (protocol number 3636/AO/15). Parental consent was obtained before enrollment of the patients in the study.

RESULTS

161 neonates having a urine sample at T_0 were recruited, of that 69 were diagnosed with BPD (42.9%) and 92 without BPD (57.1%). Among neonates with BPD, 33 (47.8%) were classified as mild BPD, 16 (23.2%) as moderate BPD, 17 (24.6%) as severe BPD and 3 (4.4%) were unclassified.

For each of the 161 neonates recruited, 7 clinical data that could potentially influence the development of BPD were recorded: sex, gestational age (GA), birth weight, intra uterine growth restriction (IUGR), premature rupture of membranes (PROM), early onset of sepsis (EOS) and antenatal steroid treatment were recorded (Table 7).

Table 7. Clinical characteristics of population (total, BPD, no BPD)

	Total 161	**BPD** 69 (42.9%)	**noBPD** 92 (57.1%)	***p-value***
Sex n (%) male female	 86 (53.4) 75 (46.6)	 41 (59.4) 28 (40.6)	 45 (48.9) 47 (51.1)	 0.186
Gestational age at birth (weeks) median (IQR)	 29 (27-31)	 26 (25-28)	 30 (29-31)	 <0.001*
Gestational age at birth (days) median (IQR)	 204 (186-215)	 183 (174-193)	 211 (205-219)	 <0.001*
Birth weight (grams) median (IQR)	1115 (800-1400)	760 (630-960)	1335 (1149-1489)	 <0.001*
IUGR n (%) yes no NA	 19 (11.9) 141 (88.1) 1	 12 (17.6) 56 (82.4) 1	 7 (7.6) 85 (92.4) -	 0.078
PROM n (%) yes no	 53 (32.9) 108 (67.1)	 16 (23.2) 53 (76.8)	 37 (40.2) 55 (59.8)	 0.023*
Antenatal steroids n (%) yes no NA	 150 (93.7) 10 (6.3) 1	 63 6 -	 87 4 1	 0.369
EOS n (%) yes no	 48 (29.8) 113 (70.2)	 27 (39.1) 42 (60.9)	 21 (22.8) 71 (77.2)	 0.025*

A training set of 81 neonates (35 BPD and 46 noBPD) and a test set of 80 neonates (34 BPD and 46 noBPD) were randomly selected stratifying the observations on the basis of GA.

Predicting BPD by means of clinical data

A RF was optimised on the training set. The optimal parameters were 1 predictor randomly sampled as candidate at each split, 800 trees and a minimum size of 10 for the terminal nodes. RF showed MCC=0.828, MCCoob=0.676 and MCCpred=0.719. The scatter plot reported in Figure 5 shows the data representation obtained by RF.

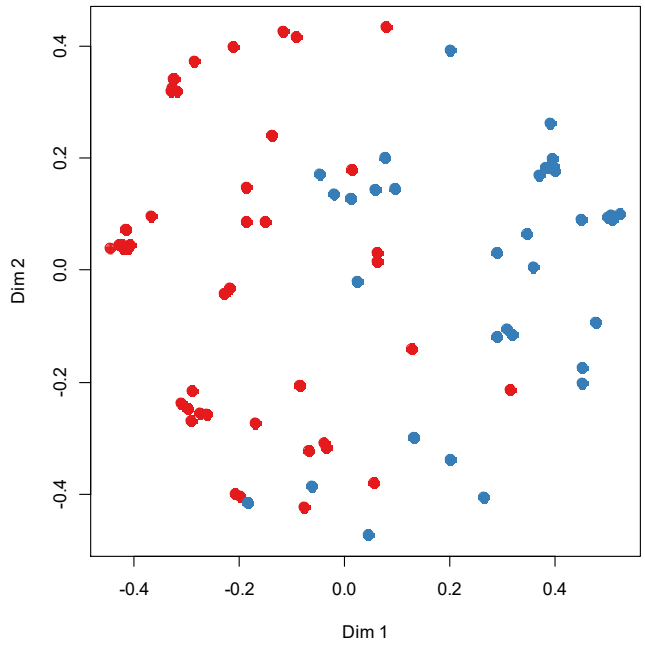

Figure 5. Clinical data: representation of the training set obtained by RF; blue circles are used for BPD group and red circles for noBPD group.

The importance of the clinical data in RF is reported in the bar plot of Figure 6.

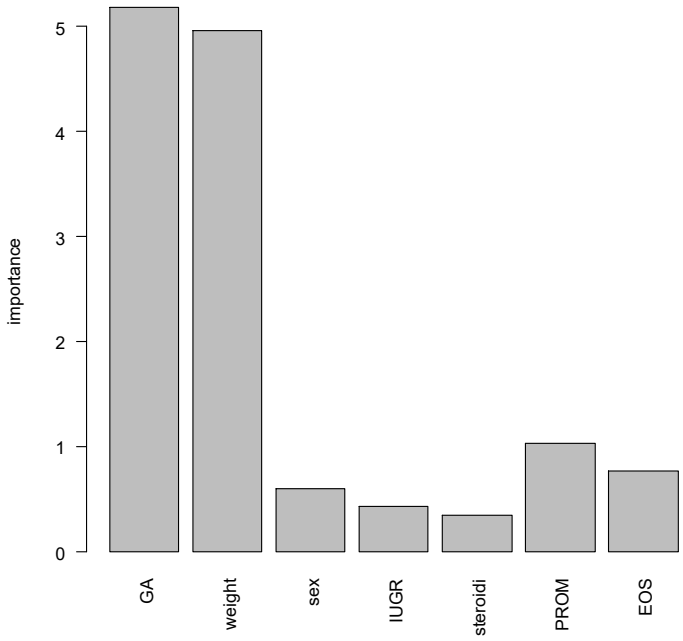

Figure 6. Clinical data: importance of the predictors in RF.

The predictors GA and weight at birth are the most important. Since GA and weight at birth are strongly correlated (ρ=0.80), we can affirm that the prediction of BPD is mainly influenced by GA. Interestingly, considering the predictions of neonates with GA≥185 days and GA<185 days the following confusion matrices were obtained (Table 8, 9)

Table 8. Prediction of BPD with GA in neonates with GA at birth≥185 days.

GA≥185	prednoBPD	pred BPD
noBPD	42	4
BPD	8	7

Table 9. Prediction of BPD with GA in neonates with GA at birth<185 days.

GA<185	prednoBPD	pred BPD
noBPD	0	0
BPD	0	19

All neonates with less than 185 days of GA were correctly predicted while most the error in classification concerned neonates developing BPD with GA≥185 days. In the following we will try to improve the quality of the predictions for GA≥185 days including the information related to the urinary metabolome.

Predicting BPD by means of urinary metabolomics data

The urinary metabolome will be investigated by PLS-methods since they allow a more clear model interpretation than RF, permitting the discovery of potential biases due to confounding factors and the correction of the models for those factors, if necessary.

The 450 time_mass variables were used to predict the outcome of BPD. Data were autoscaled. A PLS2C model was optimized on the training set by 21-repeated 5-fold cross-validation. The model showed 2 score components, MCC=0.723, MCCcv=0.468 and MCCpred=0.535. The ROC curves estimated by cross-validation are reported in Figure 7. The mean AUC in cross-validation was 0.804. The model performed worse than RF built on the clinical data.

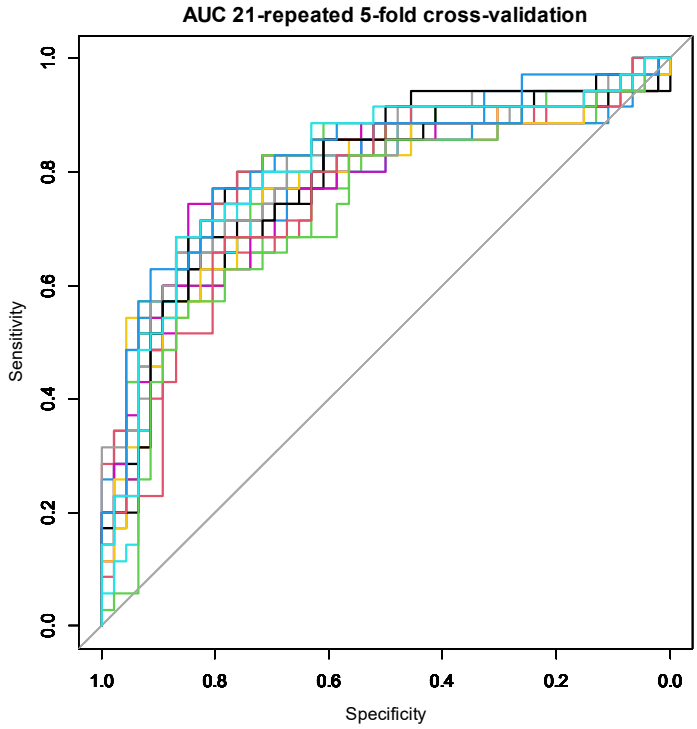

Figure 7. Metabolomics data: ROC curves under 21-repeated 5-fold cross-validation.

The most relevant annotated metabolites associated to the outcome of BPD and discovered by stability selection are reported in Table 10.

Table 10. Relevant metabolites discovered by stability selection; m/z is the mass to charge ratio, Rt is the retention time, level is the annotation level [5], HMDB is the identifier of the metabolite in the Human Metabolome Database and FC[BPD/noBPD] is the fold change measured as ratio between the median of the metabolite in the BPD group and the median in the noBPD group.

ID	m/z	Rt	annotation	level	HMDB	FC[BPD/noBPD]
POS7328	218.1393	1.236	Propionylcarnitine	1	HMDB00824	3.55
POS77	246.1707	3.320	Valerylcarnitine	1	HMDB13128	2.24
POS6591	204.1239	0.697	L-Acetylcarnitine	1	HMDB00201	2.53
POS6432	232.1551	2.388	Butyrylcarnitine	1	HMDB02013	1.74
POS105	245.0775	0.708	Pseudouridine	1	HMDB00767	1.09
POS105	245.0775	0.708	Uridine	1	HMDB00296	1.09
POS5956	257.0610	4.634	4-Phenyl-1H,3H-naphtho[1,8-cd]pyran-1,3-dione	3	HMDB41120	1.39

The predictive component of the model resulted to be strongly correlated to GA (ρ=-0.67) and weakly correlated to sex (ρ=0.24). In other words, the urinary metabolome contained information useful to predict BPD, but part of that information explained also GA and sex. If the PLS2C model is corrected for GA and sex constraining the predictive component to be orthogonal to GA and sex, the performances of the model become MCC=0.415, MCCcv=0.117, MCCpred=0.124 and mean AUC in cross-validation equal to 0.560. Then, the model becomes unsatisfactory in predicting BPD when corrected for GA. We can conclude that the effect of GA on the urinary metabolome is the main responsible of the predictive power of the PLS2C model based on the metabolic content of the urines at birth.

To improve the predictions based on the clinical data when GA≥185 days, the BPD and the noBPD observations of the training set were matched [5] in order to have no significant differences at the level of the clinical data. Assuming α=0.05, 11 observations were selected from the BPD group and 11 observations from the noBPD group. The selected subset was investigated by PLS2C with stability selection (200 random subsamples) for discovering the most relevant predictors capable to distinguish the two groups in the selected range of GA, being the PLS2C models independent of GA. In Figure 8, the ROC curves for the out-of-bag prediction are reported. The 10^{th} and the 90^{th} of the AUC were 0.500 and 0.868, respectively.

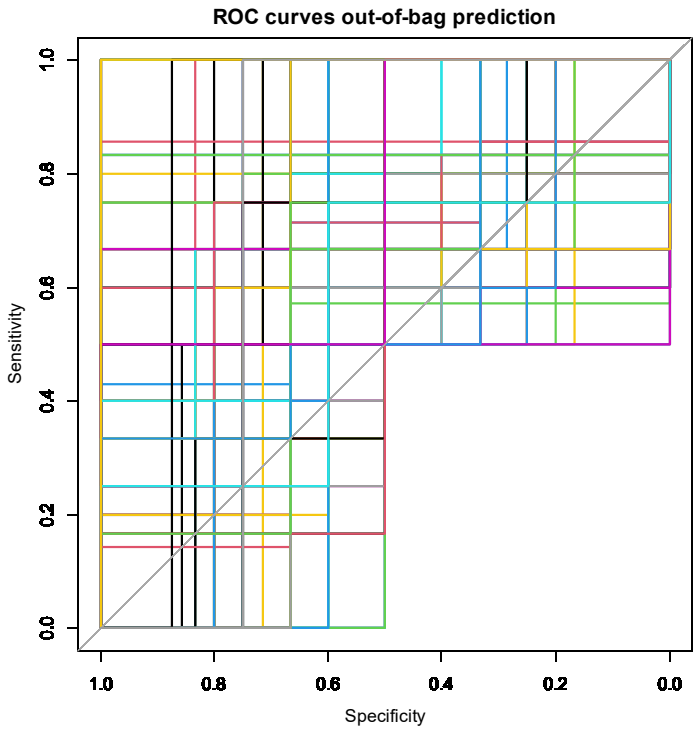

Figure 8. Mebolomics data: stability selection; ROC curves calculated during the out-of-bag prediction.

Assuming α=0.05, the 12 metabolites reported in Table 11 resulted to be relevant. Specifically, 4 metabolites were annotated at level 1.

Table 11. Relevant metabolites discovered by stability selection; m/z is the mass to charge ratio, Rt is the retention time, level is the annotation level [5], HMDB is the identifier of the metabolite in the Human Metabolome Database and FC[BPD/noBPD] is the fold change measured as ratio between the median of the metabolite in the BPD group and the median in the noBPD group.

ID	m/z	Rt	annotation	level	HMDB	FC[BPD/noBPD]
POS51	286.2020	4.985		4		1.88
POS117	302.1974	4.163	12-HEPE	3	HMDB10202	1.26
POS6931	287.1185	4.456		4		0.90
POS875	206.0487	0.770	L-Cystathionine	1	HMDB00099	1.40
POS5918	241.1554	1.042		4		1.01
POS7048	229.1552	0.781	L-isoleucyl-L-proline	3	HMDB11174	1.05
POS46	296.1500	2.833	Histidinyl-Tryptophan	3	HMDB28896	0.86
POS6873	392.1279	4.979		4		0.68
POS7188	135.0444	4.179	p-Hydroxyphenylacetic acid	1	HMDB00020	0.45
POS6844	203.0821	1.483	5-Hydroxy-L-tryptophan	3	HMDB00472	0.81
POS77	246.1707	3.320	Isovalerylcarnitine	1	HMDB00688	2.24
POS6186	132.0765	0.556	Creatine	1	HMDB00064	3.21

Predicting BPD by means of clinical data and urinary metabolomics data

The 12 metabolites discovered to be relevant in distinguishing BPD and noBPD groups when the bias due to GA was removed, were joined to the clinical data obtaining a data set composed of 19 predictors.

The optimal parameters of the RF built on the training set were 2 predictors randomly sampled as candidate at each split, 1200 trees and a minimum size of 5 for the terminal nodes. RF showed MCC=1.000, MCCoob=0.752 and MCCpred=0.744.The scatter plot reported in Figure 9 shows the data representation obtained by RF.

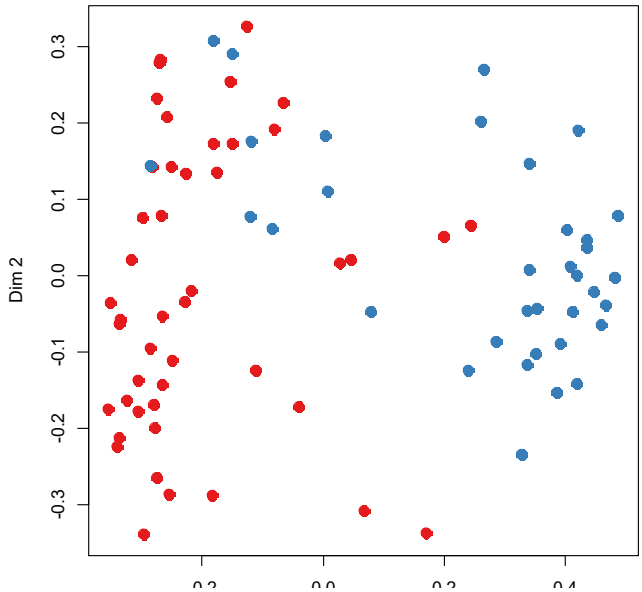

Figure 9. Clinical and metabolomics data: representation of the training set obtained by RF; blue circles are used for BPD group and red circles for noBPD group.

The importance of the predictors in RF is reported in the bar plot of Figure 10. It is worth noting that GA and weight at birth are the predictors with the highest importance, but that the contribution of the metabolites is not negligible.

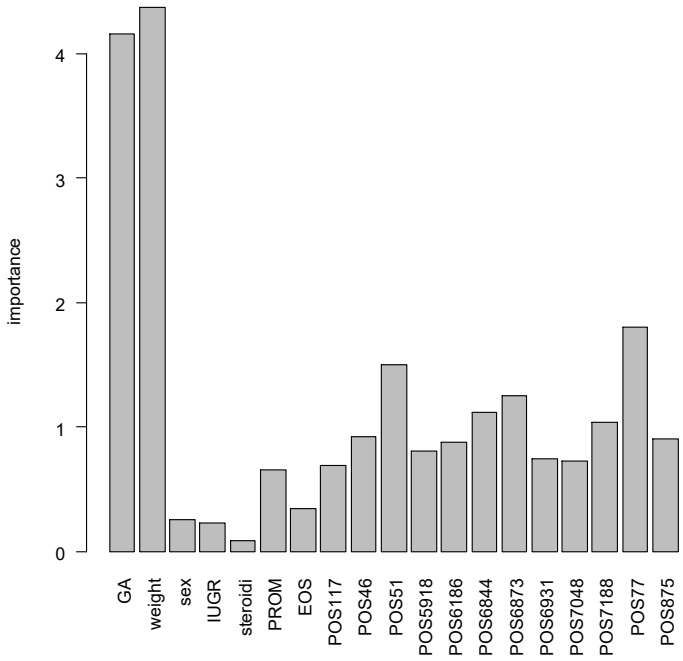

Figure 10. Clinical and metabolomics data: importance of the predictors in RF.

Indeed, the predictions were improved with respect to the use of only clinical data. The MCC values calculated for the out-of-bag prediction during model optimization for RFs built considering only clinical data and clinical data joined to the selected metabolites are reported as box plots in Figure 11. As one can observe, combining clinical data and metabolite concentration the performance in prediction were improved.

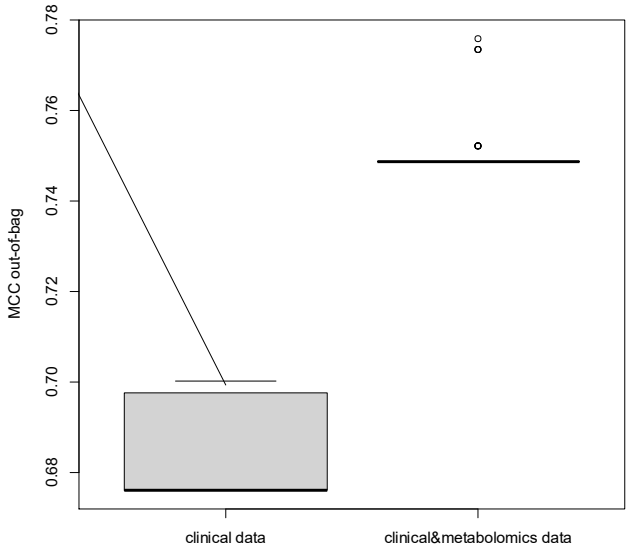

Figure 11. MCC calculated for the out-of-bag prediction during model optimization.

Moreover, considering the predictions of neonates with GA≥185 days and GA<185 days the following confusion matrices were obtained (Table 12, 13).

Table 12. Prediction of BPD using clinical and metabolomic data in neonates with GA at birth≥185 days.

GA≥185	prednoBPD	pred BPD
noBPD	43	3
BPD	7	8

Table 13. Prediction of BPD using clinical and metabolomic data in neonates with GA at birth<185 days.

GA<185	prednoBPD	pred BPD
noBPD	0	0
BPD	0	19

The 90.0% of the observations predicted to be BPD were correctly classified as BPD, whereas 86.0% of the observations predicted noBPD were correctly classified. Using only clinical data, the rate were 86.7% and 84.0%, respectively.

DISCUSSION

BPD is the main chronic respiratory disease that occurs after preterm birth and one of the major complications of prematurity. [1] The long-term consequences, both respiratory and neurological, have a significant impact on the quality of life of survivors.

Unfortunately, to date, BPD therapy is only supportive and there is no specific treatment once the disease has established. Therefore it is of fundamental importance try to prevent the onset of the disease in subjects at risk. To date, there are no biomarkers in clinical practice that allow to understand which newborns will develop BPD.

In this context, this study aimed to identify, through the metabolomic approach, urinary biomarkers at birth capable of predicting the development of BPD. Moreover, the secondary aim was to characterized the metabolome of BPD patients at birth in order to investigate whether altered metabolic pathways are still identifiable in the pathogenesis of BPD. In fact, the metabolomic approach is not influenced by a priori hypothesis, so it allows to identify new pathologic processes when the pathophysiology of the condition is not yet fully understood.

Analyzing the clinical variables of the newborns with BPD compared to controls in our sample, we found that the gestational age at birth and the birth weight were significantly lower in the BPD group, with a median GA at birth of 26 weeks in the BPD group and 30 weeks in the control group. It is well known that the incidence of BPD is inversely related to gestational age at birth and birth weight. [3] [27] [32] In fact, in the pathogenesis of BPD, prematurity is an indispensable condition and the lower the gestational age at birth, the more precociously the arrest of pulmonary development will occur, increasing the probability of the onset of the disease. [21]

The presence of early onset neonatal sepsis was also found to be greater in the BPD group. This data confirms what is known in the literature, that systemic and pulmonary infections are associated with a greater risk of BPD. [43] In our sample, however, no differences were found between the two groups as regards the presence of IUGR, in contrast to what is reported in literature, namely that IUGR is an independent risk factor for the development of BPD. [22]

Applying the metabolomic approach, the comparison of early urinary metabolome between our group of newborns who subsequently developed BPD and controls revealed an interesting clustering

of metabolites. In particular, 6 discriminant metabolites in urine were annotated with level 1 (identified compounds): L-Acetylcarnitine (C2-carnitine), Propionylcarnitine (C3-carnitine), Butyrylcarnitine (C4-carnitine), Valerylcarnitine (C5-carnitine), Uridine and Pseudouridine. All these metabolites were found in higher levels in the BPD group.

Four of the 6 identified metabolites (L-Acetylcarnitine, Propionylcarnitine, Butyrylcarnitine, Valerylcarnitine) belong to the short-chain Acylcarnitines (ACs) class. In particular, L-Acetylcarnitine is an acetic acid ester of carnitine, Propionylcarnitine is an propanoic acid ester of carnitine, Valerylcarnitine is a valeric acid ester of carnitine [87] and Butyrylcarnitine is an optically active form of O-butanoylcarnitine which is an O-butanoyl derivative of carnitine. [99] ACs are released during β-oxidation of fatty acids and their role is to transport acyl-groups (organic acids and fatty acids) from the cytoplasm into the mitochondria so that they can be broken down to produce energy (Figure 12).

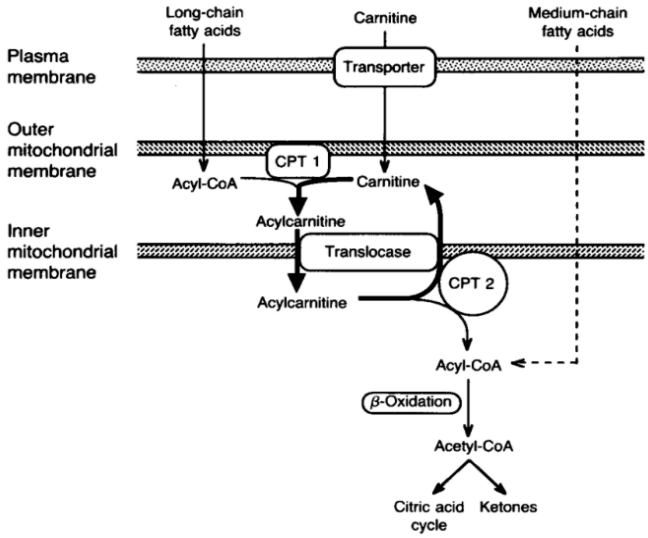

Figure 12. The role of Acylcarnitine in the Carnitine cycle of mitochondrial oxidation of long-chain fatty acids. [114]

According to a recent review, [100] ACs can be classified into 9 different categories according to the type and size of their acyl group. The short-chain ACs, to which the 4 metabolites identified in our sample belong, have acyl groups from two to five carbon atoms (C2-C5) [87]. Short-chain ACs constitute 90–95% of the total plasma ACs. [101] Mitochondrial beta-oxidation of fatty acids and the accumulation of the metabolites of this metabolic pathway, including ACs, increase the production of oxygen free radicals (ROS) and this generates a cellular environment of oxidative stress. [100] These data confirm the hypothesis already reported in literature regarding the role of exposure to oxidative stress as a risk factor for the development of BPD. [85] Also Baraldi et al. showed that infants predisposed to developing BPD has an altered lipid metabolism even if before birth, in fact they found higher levels of some hydroxy fatty acids and oxy fatty acids in amniotic fluid. They concluded that fetus predisposed to BPD has higher levels of oxidative stress already present in utero. [90] The high levels of ACs found in our early urine sample seem to indicate that exposure to a cellular environment of oxidative stress continues even after birth in the first days of life in infants who will then develop BPD. Also Piersigilli et al. found an increase in ACs (C16-OH and C18: 1-OH) in their metabolomic analysis of tracheal aspirates collected

in the first week of life in preterm infants who subsequently developed BPD. In particular, ACs were more altered in more preterm infants [83]. This seems to confirm that oxidative stress is an important pathogenetic mechanism in the development of BPD.

Uridine is a member of the class of compounds of pyrimidine nucleosides. More specifically, uridine is a nucleoside consisting of uracil and D-ribose and it is a component of RNA. Uridine can be found in most of human biofluids, including urine, breast milk, cerebrospinal fluid and blood. Within the cell, uridine is primarily located in the mitochondria, in the nucleus and the lysosome and it can also be found in the extracellular space. [87] Pseudouridine is the first modified nucleoside discovered and, specifically, it has uracil attached via a carbon-carbon instead of a nitrogen-carbon glycosidic bond to the ribofuranose. It is the most prevalent of the over one hundred different modified nucleosides found in RNA. [87] Pseudouridylation of uridine in RNAs is necessary for efficient mRNA splicing, especially in condition of cellular stress. [102]

Nucleosides are mainly known for their role as building blocks of DNA and RNA nucleic acids. However, they are also implicated in various cellular processes, such as cell signalling, enzyme regulation and metabolism. Modified nucleoside analogues have been used for many years as antitumor and antiviral therapy and recently, due to their immunomodulatory effect (in particular of adenosine) in asthma therapy. *In vivo* in a study on a mice animal model of asthma and *in vitro*, uridine was shown to be able to reduce lung inflammation. [103] [104] In particular it was highlighted a reduction in the total cell count of bronchoalveolar lavage and in pro-inflammatory cytokines such as IL-4, IL-6, IL-13 after treatment with uridine. [104] In an animal model of pulmonary fibrosis, uridine administration, in addition to anti-inflammatory capacity, also showed antifibrotic properties, reducing collagen deposition in the lung interstitium. [105] In fact, uridine inhibits collagen and TGF-ß synthesis by primary lung fibroblasts, the release of pro-inflammatory cytokines by human lung epithelial cells, as well as the production of reactive oxygen species by human neutrophils. [105] Another positive effect of uridine on lungs is the protective action against hypoxia-induced pulmonary injury. Rozova et al. in fact showed that uridine administration to animals prior to hypoxia exposure decreased the number of mitochondria with altered ultrastructure, prevented the hypoxia-induced mitochondrial damage and protected the epithelial, interstitial and endothelial layers of the air-blood barrier from the hypoxia-induced hyperhydration. [106]

Inflammation plays a central role in the pathogenesis of BPD and many pro-inflammatory cytokines have been found elevated both in amniotic fluid, [23] blood and tracheal aspirate of newborns with

BPD, including interleukin 6. [54] Furthermore, the fibrotic remodeling of the lung in the most advanced stages of BPD and the frequent episodes of hypoxia associated to respiratory failure that accompany BPD are also part of its natural history. Therefore we hypothesize that the high uridine levels found in BPD neonates can be interpreted as a compensatory mechanism of the organism in response to inflammation, hypoxia and fibrotic remodeling, carried out through its anti-inflammatory and antifibrotic effect and its protection ability against hypoxia-induced damage.

The role of uridine in inflammation associated with BPD is also demonstrated by the study of pharmacometabolomics carried out by Lewis et al., in which they showed that urinary uridine and pseudouridine levels drop after treatment with systemic dexamethasone. Furthermore, in their study uridine well correlates with the degree of clinical response to steroid, in particular the greater the drop in urinary concentration the better the response. [51]

From the analysis of our sample it emerged that the urinary metabolome significantly correlates with gestation age. The dependence of the metabolome on gestational age, as well as on other factors such as sex and the treatment carried out, is an emerging evidence. [107] [108]

Ernst et al., using an untargeted liquid chromatography-tandem mass spectrometry metabolomics analysis on dried blood spots from the Neonatal Screening Biobank, compared the blood metabolome of premature infants (28-36 weeks' GA) with that of term infants (37-42 weeks' GA). They found that a total of 1459 metabolites of over 9000 (about 16%) measured were significantly correlated with gestational age. These metabolites belong to bile acids, carnitines, polyamines, amino acid-derived compounds, nucleotides, phosphatidylcholines and dipeptides and metabolites related to treatment (antibiotics and caffeine). [107] This difference on metabolome in preterm infants can be associated with lowered maturation of metabolic pathways, increased catabolic stress and specific treatment required (caffeine, parenteral nutrition, antibiotics). [108]

Also Caterino et al. found that the blood metabolome differs among very preterm infants compared to moderately-late preterm infants and that also the differences in metabolome between sex change with gestational age. For example, male and female very premature infants diverged in amino acids but not in Acylcarnitines, whereas the opposite was observed in moderate-late preterm infants. [108]

However, to the best of our knowledge, no study has evaluated how metabolome changes in urine and in extremely preterm infants (<28 weeks' GA), which are included in our study. Therefore, we compared the metabolome of BPD infants and controls of 2 homogeneous populations for GA to remove the effect of gestational age on the metabolome. From this analysis we identified with level

1 of the annotation 3 interesting metabolites that discriminate patients with BPD from controls: Isovalerylcarnitine, L-Cystathionine, Creatine and p-Hydroxyphenylacetic acid.

Isovalerylcarnitine is a C5-acylcarnitine having isovaleryl as the acyl substituent. It belongs to the short-chain ACs, [99] as the 3 metabolites previously identified in this study, and it was also found in higher concentrations in the BPD group. This result seems to confirm the role of short-chain Acs in the pathogenetic mechanism of BPD and, among all ACs identified in this study, Isovalerylcarnitine is able to discriminate infants predisposed to develop BPD from controls regardless of gestational age. Also Piersigilli et al. found that the ability of ACs in tracheal aspirate samples to discriminate infants predisposed to BPD from controls depends on gestation age, particularly C18: 1-OH was mainly abundant in the BPD group of infants <27 weeks' GA. [85]

L-Cystathionine is a dipeptide formed by serine and homocysteine (Figure 13) and it is the precursor of cysteine, through the enzyme γ-cystathionase. [109] Glutathione is the most important intracellular antioxidant of free radicals and other oxygen reactive species and it requires cysteine for its synthesis (Figure 13). [109] L-methionine S-adenosyltransferase is inactivated when gluthatione is depleted, as happens for consumption in situations of oxidative stress. The reduction of this enzyme causes, in cascade, the reduction of the conversion of homocysteine to cystathione and consequently of cysteine, causing a vicious circle as cysteine is necessary for the synthesis of glutathione itself. [109] Therefore, under conditions of oxidative stress, as occurs in BPD, a reduction in glutathione and cysteine levels would be expected. In fact, in various metabolomics studies applied to BPD, reduced metabolites associated with this pathway have been found, suggesting a reduced presence of glutathione in patients with BPD. Carraro et. al, for example, found in exhaled breath condensate in adolescents with BPD, reduced concentrations of S-adenosyl-methionine, which is a precursor of the glutathione. [91] Also Pintus et al. found in the urinary metabolome of BPD neonates of 7 days of life, reduced concentration of

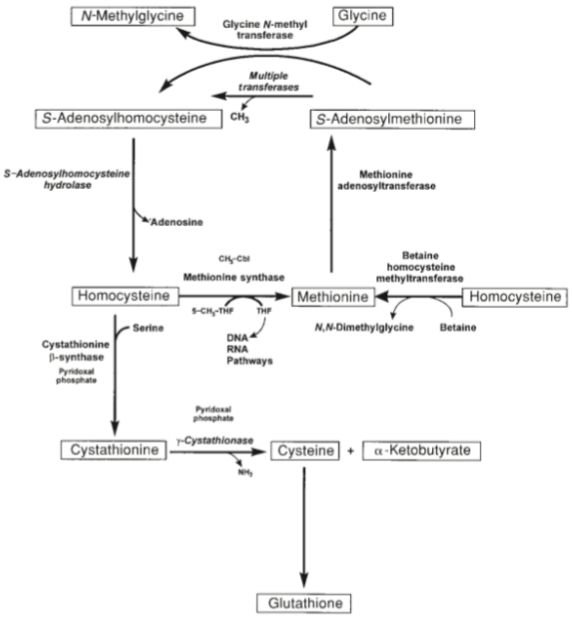

Figure 13. Pathways of cystathionine metabolism. [109]

glycine, which also play an important role in glutathione synthesis. [89] However, on the contrary, in our study we found higher levels of L-cystathionine, which suggests higher levels of glutathione, in the BPD group than in the controls. This can be interpreted as an increase in the demand for antioxidants by the organism subjected to greater oxidative stress, as in BPD. Another possible interpretation of the increase in cystathionine levels found in our BPD population, could be that in the first 1-2 weeks of life, the expression of γ-cystathionase is reduced, [109] as occurs in newborn animals, causing an accumulation of cystathionine that fails to convert into cysteine, which is in turn more required in infants with oxidative stress linked to the predisposition to BPD. In fact, the urine samples in our study are very early (within 48 hours of life), therefore γ-cystationinase may still be poorly expressed, while in the study of Pintus et al. urine samples were collected at 7 days of life and in the study of Carraro et al. the population was made up of adolescents. [89] [91]

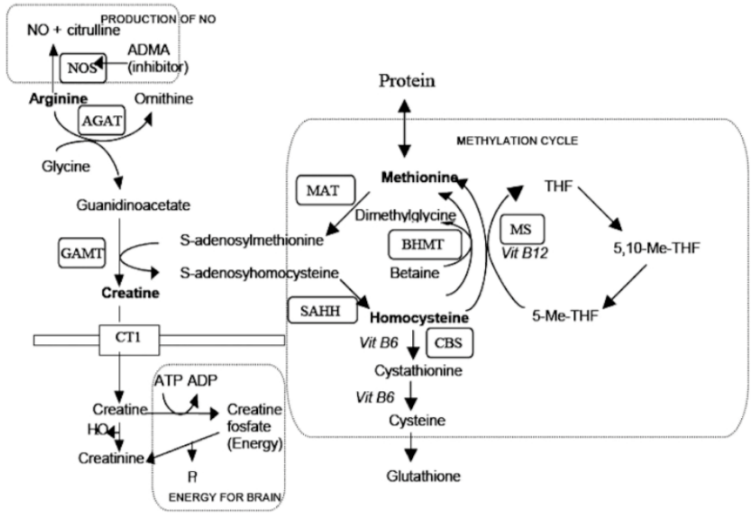

Figure 14. Relationship between the metabolic pathway of methionine-cystathionine and of creatine. [115]

Moreover, S-adenosylmethionine, which is implicated in the methionine-cystathionine pathway, is the source of methyl groups important in the synthesis of creatine (Figure 14), another metabolite found in higher levels in our BPD group. Creatine is an endogenous amino acid present primarily in muscle cells. It is important for energy storage and it serves as a phosphate donor in the conversion of ADP to ATP and supplies energy necessary for muscle contraction. [99] Creatine is predominantly known for the role as ergogenic aids, however it has several health and potential therapeutic benefits, including a role as an antioxidant. [110] In our sample, higher creatine levels were found in patients predisposed to BPD than in controls, probably related to the increase in cystathionine and its metabolic pathway. In any case, further studies are needed to clarify how methionine-cystationine-glutathione pathway and creatine role fit into the pathogenesis of BPD.

p-Hydroxyphenylacetic acid, also known as 4-Hydroxyphenylacetic acid (4-HPA), belong to the compounds of phenolic acids that are aromatic acids that contain a phenol ring and at least one

organic carboxylic acid group. [111] P-Hydroxyphenylacetic acid is also a microbial metabolite produced by Acinetobacter, Clostridium, Klebsiella, Pseudomonas, and Proteus. Higher levels of this metabolite are associated with an overgrowth of small intestinal bacteria from Clostridia species. [87] In lung tissues, 4-HPA attenuated hypoxia, inflammation, vascular leak and edema. In primary rat alveolar epithelial cells, 4-HPA decreased hypertonicity- and hypoxia-induced (HIF)-1α protein levels which causes lowering inflammatory cytokines levels and monolayer permeability. [112] In our sample, infants with predisposition to BPD have lower levels of 4-HPA. Given the protective effects it exerts on the lung, its reduction can be explained as a reduced ability of these infants to respond to the pathogenic noxae causing lung damage. 4-HPA also originates from the intestinal microbial flora, suggesting a possible intestinal dysbiosis in these newborns. Recently, in fact, the role of microbiome alterations in the pathogenesis of a large number of diseases, including BPD, is emerging in literature. [47] [49]

In the last part of our work we calculated the predictive capacity on BPD of clinical variables alone, (the most important of which is the gestational age at birth) and of clinical variables associated with the analysis of identified metabolites. We found that the analysis of urinary metabolites increases the predictivity of BPD in preterm infants at birth. In particular, the sensitivity increases from 86.7% to 90.0% and the specificity from 84.0% to 86.0%. This confirms a possible role of the identified metabolites in improving the early diagnosis of BPD.

The strengths of this study are the high sample size, higher than other metabolomics studies present in literature on the same topic, and consequently the possibility of using a validation test set that provides robustness to statistical analyzes.

A limit of our study is the low number of control infants without BPD extremely preterm, in particular <26 weeks' GA.

Future prospects

In the Veneto region, with the decree of the regional council number 2013/1308, [113] the expanded neonatal metabolic screening has been implemented since 2014 in order to identify some inherited metabolic diseases already at birth. In that screening, some Acylcarnitines are tested on blood spots at 48 hours of life, in particular Propionylcarnitine, Butyrylcarnitine, Isovalerylcarnitine are included. Since in the present study these 3 Acylcarnitines in urine have been shown to increase the predictivity of BPD in preterm infants, this could lead to consider this diagnostic test already in use to evaluate

the predisposition to BPD of the preterm newborn through the same blood sample. However, it will first be necessary verify that the level of these metabolites is also confirmed to be elevated in the plasma of newborns predisposed to BPD, then to identify the normal concentration of Acylcarnitines in the blood of preterm infants, also in relation to the different gestational ages, and the relative cut-off value that indicates the predisposition to BPD.

This study is part of an important line of research that aims to improve the predictive ability of the diagnosis of BPD, in order to identify the subjects most at risk already in the perinatal period. Such infants, in the next future, may be possible candidates for the administration of new therapies such as Mesenchymal stem/stromal cells (MSCs)-derived extracellular vesicles (EVs), currently still in the experimental phase, which in the animal model have shown promise results in reducing the development of BPD. [17]

CONCLUSIONS

BPD is one of the major complications of prematurity and its incidence is still increasing. Unfortunately, there is currently no specific treatment, therefore it is important to identify infants at risk already in the first days of life to try to prevent the development of the disease. The metabolomic approach on urine samples collected within 48 hours of life revealed an interesting clustering of metabolites that discriminate between preterm infants at risk of developing BPD versus infants who will not develop it. The main metabolites identified are molecules belonging to the Acylcarnitine group, Uridine, Pseudouridine, L-Cystathionine, Creatinine and p-Hydroxyphenylacetic acid. The analysis of identified metabolites, associated with clinical data, especially gestational age at birth, increases the prediction of the development of BPD in preterm infants. In the next future, early detection of infants at risk of developing BPD may be useful for the administration of targeted therapies.

REFERENCES

[1] E. Baraldi and M. Filippone, "Chronic Lung Disease after Premature Birth," *N Engl J Med,* vol. 357, pp. 1946-1955, 2007.

[2] W. Nortway, R. Rosan and D. Porter, "Pulmonary disease following respirator therapy of hyaline-membrane disease," *The New England journal of Medicine,* vol. 267, no. 7, pp. 357-368, 1967.

[3] B. Stoll, N. Hansen and E. Bell, "Trends in Care Practices, Morbidity, and Mortality of Extremely Preterm neonates," *JAMA,* vol. 314, no. 10, pp. 1039-1051, 2015.

[4] A. Hislop, J. Wigglesworth and R. Desai, "Alveolar development in the human fetus and infant.," *Early Hum Dev,* vol. 13, pp. 1-11, 1986.

[5] A. Jobe, "The New BPD: An Arrest of Lung Development," *Pediatr Res,* vol. 46, no. 6, pp. 641-643, 1999.

[6] W. Laocharoensap, S. Gage, P. Kan, J. Profit, G. Shaw, J. Gould, D. Stevenson, H. O'Brodovich and H. Lee, "Hospital Variation and Risk Factors for Bronchopulmonary Dysplasiain a Population_Based Cohort," *JAMA Pediatr,* vol. 169, no. 2, p. e143676, 2015.

[7] J. Horbar, E. Edwards, L. Greenberg, K. Morrow, R. Soll, M. Buss-Frank and J. Buzas, "Variation in Performance of Neonatal Intensive Care Units in United States," *JAMA Pediatr,* vol. 171, no. 3, p. e164396, 2017.

[8] C. Siffel, K. Kistler, J. Lewis and S. Sarda, "Global incidence of bronchopulmonary dysplasia among extremely preterm infants: a systematic literature review," *J Matern Fetal Neonatal Med,* vol. 34, no. 11, pp. 1721-1731, 2021.

[9] W. Tooley, "Epidemiology of bronchopulmonary dysplasia," *J Pediatr,* vol. 95, no. 5, pp. 851-855, 1979.

[10] A. Shennan, M. Dunn, A. Ohlsson, K. Lennox and E. Hoskins, "Abnormal pulmonary outcomes in premature infants: prediction from oxygen requirement in the neonatl period," *Pediatrics,* vol. 82, no. 4, pp. 527-532, 1988.

[11] A. Jobe and E. Bancalari, "Bronchopulmonary Dysplasia," *Am J Respir Crit Care Med,* vol. 163, p. 1723–1729, 2001.

[12] M. Walsh, Q. Yao, P. Gettner, E. Hale, M. Collins, A. Hensman, R. Everette, N. Peters, N. Miller, G. Muran, K. Auten, N. Newman, G. Rowan, C. Grisby, K. Arnell, L. Miller, B. Ball and G. McDavid, "Impact of a Physiological Definition of Bruncopulmonary Dysplasia Rates," *Pediatrics,* vol. 114, no. 5, pp. 1305-1311, 2004.

[13] B. Poindexter, R. Feng, B. Schmidt, J. Aschner, R. Ballard, A. Hamvas, A. Reynolds, P. Shaw and A. Jobe, "Comparisons and Limitations of Current Definitions of Bruncopulmonary Dysplasia for the Prematurity and Respiratory Outcomes Program," *Ann Am Thorac Soc,* vol. 12, no. 12, pp. 1822-1830, 2015.

[14] R. Higgins, A. Jobe , M. Koso-Thomas, E. Bancalari, R. Viscardi, T. Hartert, R. Ryan, S. Kallapur, R. Steinhorn, G. Konduri, S. Davis, B. Thebaud, R. Clyman, J. Collaco, C. Martin, J. Woods, N. Finner and T. Raju, "Bronchopulmonary Dysplasia: Executive Summary of a Workshop," *J Pediatr,* vol. 197, pp. 300-308, 2018.

[15] E. Jensen, K. Dysart, M. Gantz, S. McDonald, N. Bamat, M. Keszler, H. Kirpalani, B. Poindexter, A. Ducan, B. Yoder, E. Eichnwald and S. DeMauro, "The Diagnosis of

Bruncopulmonary Dysplasia in Very Preterm Infants. An Evidence-Based Approach," *Am J Respir Crit Care Med,* vol. 200, no. 6, pp. 751-759, 2019.

[16] P. Burri, "Fetal e Postanatal Development of the Lung," *Ann Rev Physiol,* vol. 46, pp. 617-628, 1984.

[17] L. Bonadies, P. Zaramella, A. Porzionato, G. Perilongo, M. Muraca and E. Baraldi, "Present and Future of Bronchopulmonary Dysplasia," *J Clin Med,* vol. 9, p. 1539, 2020.

[18] A. Jobe, "Mechanisms of Lung Injury and Bruncopulmonary Dysplasia," *Am J Perinatol,* vol. 33, pp. 1076-1078, 2016.

[19] V. Bhandari, M. Bizzarro, A. Shetty, X. Zhong, G. Page, H. Zhang, L. Ment and J. Gruen, "Familial an Genetics Susceptibility to Major Neonatal Morbidities in Preterm Twins," *Pediatrics,* vol. 117, no. 6, pp. 1901-1906, 2006.

[20] V. Bhandari and J. Gruen, "The Genetics of Bronchopulmonary Dysplasia," *Semin Perinatol,* vol. 30, pp. 185-191, 2006.

[21] E. Bancalari and D. Jain, "Bronchopulmonary Dysplasia: 50 Years after the Original Description," *Neonatology,* vol. 115, p. 384–391, 2019.

[22] C. Bose, L. Van Marter, M. Laugnon, T. O'Shea, E. Allred, P. Karna, R. Ehrenkranz, K. Boggess and A. Leviton, "Fetal growth restriction and risk of chronic lung disease among infants born before the 28th week of gestation," *Pediatrics,* vol. 124, no. 3, pp. e450-e458, 2009.

[23] J. Lee, K. Oh, H. Yang, J. Park, R. Romero and B. Yoon, "The importance of intra-amniotic inflammation in the subsequent development of atypical chronic lung disease," *J Matern Fetal Neonatal Med,* vol. 22, no. 10, pp. 917-923, 2009.

[24] B. Yon, R. Romero, J. Jun, K. Park, J. Park, F. Ghezzi and B. Kim, "Amniotic fluid cytokines (tumor necrosis factor-a, interleukin-1b, and interleukin-8) and the risk for the development of bronchopulmonary dysplasia," *Am J Obstst Gynecol,* vol. 177, no. 4, pp. 825-830, 1997.

[25] A. Ballard, L. Mallett, J. Pruszynski and J. Cantey, "Chorioamnionitis and subsequent brochopulmonary dysplasia in very-low-birth weight infants: a 25-years cohort," *J Perinatol,* vol. 36, no. 12, pp. 1045-1048, 2016.

[26] M. Lahra, P. Beeby and H. Jeffery, "Intrauterine Inflammation, Neonatal Sepsis, and Chronic Lung Disease: A 13-Year hospital Cihirt Study," *Pediatrics,* vol. 123, pp. 1314-1319, 2009.

[27] H. Torchin, P. Ancel, F. Goffinet, J. Hascoet, P. Truffert, D. Tran, C. Lebeaux and P. Jarreau, "Placental Complications and Brunchopulmonary Dysplasia: EPIPAGE-2 Cohort Study," *Pediatrics,* vol. 137, no. 3, p. e20152163, 2016.

[28] R. Antonucci, P. Contu, A. Porcella, C. Atzeni and S. Chiappe, "Intrauterine smoke exposure: a new risk factor for bronchopumonary dysplasia?," *J Perinat Med,* vol. 32, pp. 272-277, 2004.

[29] M. Wagijo, A. Sheikh, L. Duijts and J. Been, "Reducing tobacco smoking and smoke exposure to prevent preterm birth and its complications," *Pediatr Respir Rev,* vol. 22, pp. 3-10, 2017.

[30] P. Carrera, C. Di Resta, C. Volonteri, E. Castiglioni, S. Bonfiglio, D. Lazarevic, D. Cittaro, E. Stupka, M. Ferrari and M. Somaschini, "Exome sequencing and pathway analysis for identification of genetic variability relevant for bronchopulmonary dysplasia (BPD) in preterm newborns: A pilot study," *Clin Chim Acta,* vol. 451, no. Pt A, pp. 39-45, 2015.

[31] J. Li, K. Yu, J. Oehlert, L. Jeliffe-Pawloski, J. Gould, D. Stevenson, M. Snyder, G. Shaw and H. O'Brodovich, "Exome Sequencing of Neonatal Blood Spots and the Identification of Genes

Implicated in Bronchopulmonary Dysplasia," *Am J Respir Crit Care Med,* vol. 192, no. 5, pp. 589-596, 2015.

[32] A. Trembath and M. Laughon, "Predictors of Bronchopulmonary Dysplasia," *Clin Perinatol,* vol. 39, no. 3, p. 585–601, 2012.

[33] M. Ito, M. Tamura and F. Namba, "Role of sex in morbidity and mortality of very premature neonates," *Pediatr Int,* vol. 59, no. 8, pp. 898-905, 2017.

[34] R. Ryan, R. Feng, C. Bazacliu, T. Ferkol, C. Ren, T. Mariani, B. Poindexter, F. Wang and P. Moore, "Black Race Is Associated with a Lower Risk of Bronchopulmonary Dysplasia," *J Pediatr,* vol. 207, pp. 130-135, 2019.

[35] A. Slutsky, "Lung Injury Caused by Mechanical Ventilation," *Chest,* vol. 116, pp. 9S-15S, 1999.

[36] A. Slutsky and V. Ranieri, "Ventilator.Induced Lung Injury," *N Eng J Med,* vol. 369, pp. 2126-2136, 2013.

[37] R. Thekkeveedu, M. Guaman and B. Shivanna, "Bronchopulmonary dysplasia: A Review of Pathogenesis and Pathophysiology," *Respir Med,* vol. 132, pp. 170-177, 2017.

[38] S. Perrone, M. Tataranno and G. Buonocore, "Oxidative Stress and Bronchopulmonary Dysplasia," *J Clin Neonatol,* vol. 1, no. 3, pp. 109-114, 2012.

[39] B. Buczynski, E. Maduekwe and M. O'Reilly, "The Role of Hyperoxia in the Pathogenesis of Experimental BPD," *Semin Perinatol,* vol. 37, no. 2, pp. 69-78, 2013.

[40] R. Delemos, J. Coalson, D. Gerstmann, T. Kuehl and D. Null, "Oxygen Toxicity in the Premature Baboon with Hyaline Membrane Disease," *Am Rev Respir Dis,* vol. 136, pp. 677-682, 1987.

[41] B. Warner, L. Stuart, R. Papes and J. Wispé, "Functional and pathological effects of prolonged hyperoxia in neonatal mice," *An J Physiol,* vol. 275, no. 1, pp. L110-L117, 1998.

[42] W. Maniscalco, R. Watkins, C. D'Angio and R. Ryan, "Hyperoxic Injury Decreases Alveolar Epithelial Cell Expression of Vascular Endothelial Growth Factor (VEGF) in Neonatal Rabbit Lung," *Am J Respir Cell Mol Biol,* vol. 16, pp. 557-567, 1997.

[43] G. Klinger, I. Levy, L. Sirota, V. Boyko, L. Lerner-Geva and B. Reichman, "Outcome of Early-Onset Sepsis in a National Cohort of Very Low Birth Weight Infants," *Pediatrics,* vol. 125, no. 4, pp. e736-e740, 2010.

[44] T. Gerhardt and E. Bancalari, "Lung Compliance in Newborns with Patent Ductus Arteriosus before and after Surgical Ligation," *Biol Neonate,* vol. 38, pp. 96-105, 1980.

[45] M. Liebowitz, R. Clyman, J. Kaempf, O. Erdeve, A. Bulbul, S. Hakansson, J. Lindqvist, A. Farooqi, A. Katheria, J. Sauberan, J. Singh, K. Nelson, A. Wickremasinghe, L. Dong, D. Hassinger, S. Aucott, M. Hayashi, A. Heuchan, W. Carey, M. Derrick and Fernan, "PDA-TOLERATE Trial: An Exploratory Randomized Controlled Trial of Treatment of Moderate-to-Large Patent Ductus Arteriosus at 1 Week of Age," *J Pediatr,* vol. 205, pp. 41-48, 2019.

[46] J. Slaughter, P. Reagan, T. Newman and M. Klebanoff, "Comparative Effectiveness Of Nonsteroidal Anti-inflammatory Drug Treatment Versus No Treatment For Patent DuctusArteriosus in Preterm Infants," *JAMA Pediatr,* vol. 171, no. 3, p. e164354, 2017.

[47] P. Lohmann, R. Luna, E. Hollister, S. Devaraj, T. Mistretta, S. Welty and J. Versalovic, "the airway microbiome of intubated premature infants: characteristics and changes that predict the development of bronchopulmonary dysplasia," *Pediatr Res,* vol. 76, no. 3, pp. 294-301, 2014.

[48] R. Martin, S. Langa, C. Reviriego, E. Jimenez, M. Marin, M. Olivares, J. Boza, J. Jimenez, L. Fernandez, J. Xaus and J. Rodriguez, "The commensal microflora of human milk: new

perspectives for food bacteriotherapy and probiotics," *Trends in Food Science & Technology,* vol. 15, no. 3-4, pp. 121-127, 2004.

[49] C. Lal, C. Travers, Z. Aghai, P. Eipers, T. Jiling, B. Halloran, W. Carlo, J. Keeley, G. Rezonzew, R. Kumar, C. Morrow, V. Bhandari and N. Ambalavanan, "The Airway Microbiome at Birth," *Sci Rep,* vol. 6, p. 31023, 2016.

[50] M. Pammi, C. Vivek Lal, B. Wagner, P. Mourani, P. Lohmann, R. Luna, A. Sisson, B. Shivanna, E. Hollister, S. Abman, J. Versalovic, G. Connett, V. Bhandari and N. Ambalavanan, "Airway Microbiome and Development of Bronchopulmonary Dysplasia in Preterm Infants: A Systematic Review," *J Pediatr,* vol. 204, pp. 126-133, 2019.

[51] T. Lewis, P. Chalise, C. Gauldin and W. Truog, "Pharmacometabolomics of Respiratory Phenotypic Response to Dexamethasone in Preterm Infants at Risk for Bronchopulmonary Dysplasia," *Clin Transl Sci,* no. 12, pp. 591-599, 2019.

[52] R. Viscardi and S. Kallapur, "Role of Ureaplasma Respiratory Tract Colonization in BPD Pathogenesis: Current Concepts and Update," *Clin Perinatol,* vol. 42, no. 4, pp. 719-738, 2015.

[53] L. Askie, B. Darlow, P. Davis, N. Finer, B. Stenson, M. Vento and R. Whyte, "Effects of targeting lower versus higher arterial oxygen saturations on death or disability in preterm infants," *Cichrane Database Syst Rev,* vol. 4, no. 4, p. CD011190, 2017.

[54] M. Gilfillan, A. Bhandari and V. Bhandari, "Diagnosis and management of bruncopulmonary dysplasia," *BMJ,* vol. 375, p. n1974, 2021.

[55] C. Klingenberg, K. Wheeler, N. McCallion, C. Morley and P. Davis, "Volume-targeted versus pressure-limited ventilation in neonates," *Cochrane Database Syst Rev,* vol. 10, no. 10, p. CD003666, 2017.

[56] J. Berger, P. Mehta, E. Bucholz, J. Dziura and V. Bhandari, "Inpact of Early Extubation and reintubation on the Incidence of Bronchiopulmonary Dysplasia in Neonates," *Am J Perinatol,* vol. 31, pp. 1063-1072, 2014.

[57] G. Schmölzer, M. Kumar, G. Pichler, K. Aziz, M. O'Reilly and P. Cheung, "Non-invasive versus invasive respiratory support in preterm infants at birth: systematic review and meta-analysis," *BMJ,* vol. 347, p. f5980, 2013.

[58] H. Kirpalani, D. Millar, B. Lemyre, B. Yoder, A. Chiu and R. Roberts, "A Trial Comparing Noninvasive Ventilation Strategies in Preterm Infants," *N Engl J Med,* vol. 369, pp. 611-620, 2013.

[59] R. Soll and E. Özek, "Prophylactic protein free synthetic surfactant for preventing morbidity and mortality in preterm infants," *Cochrande database Sysr Rev,* vol. 1, p. CD001079, 2010.

[60] T. Yeh, C. Chen, S. Wu, Z. Husan, T. Li, W. Hsieh, C. Tsai and H. Lin, "Intratracheal Administration of Budesonide/Surfactant to Prevent Bronchopulmonary Dysplasia," *Am J Respir Crit Care Med,* vol. 193, no. 1, pp. 86-95, 2016.

[61] T. Isayama, H. Iwami, S. McDonald and J. Beyene, "Association of Noninvasive Ventilation Strategies With Mortality and Bronchopulmonary Dysplasia Among Preterm Infants A Systematic Review and Meta-analysis," *JAMA,* vol. 316, no. 6, pp. 611-624, 2016.

[62] B. Schmidt, R. Roberts, P. Davis, L. Doyle, K. Barrigton, A. Ohlsson, A. Solimano and W. Tin, "Caffeine Therapy for Apnea of prematurity," *N Engl J Med,* vol. 354, pp. 2112-2121, 2006.

[63] V. Vadakkencherry Ramaswamy, T. Bandyopadhyay, D. Nanda, P. Bandiya, J. Ahmed, A. Garg, C. Roehr and S. Nangia, "Assessment of Postnatal Corticosteroids for the Prevention of Bronchopulmonary Dysplasia in Preterm Neonates: A Systematic Review and Network Meta-analysis," *JAMA Pediatr,* vol. 175, no. 6, p. e206826, 2021.

[64] S. Araki, S. Kato, F. Namba and E. Ota, "Vitamin A to prevent bronchopulmonary dysplasia in extremely low birth weight infants: a systematic review and meta-analysis," *PLoS ONE,* vol. 13, no. 11, p. e0207730, 2018.

[65] J. Uberos, S. Jimenez-Montilla, M. Molina-Oya and J. Garcia-Serrano, "Early energy restriction in premature infants and bronchopulmonary dysplasia: a cohort study," *Br J Nutr,* vol. 123, no. 9, pp. 1024-1031, 2020.

[66] O. Dicky, V. Ehlinger, N. Montjaux, G. Gremmo-Feger, J. Sizun, J. Rozé, C. Arnauld and C. Casper, "Policy of feeding very preterm infants with their mother's own fresh expressed milk was associated with a reduced risk of bronchopulmonary dysplasia," *Acta Pediatr,* vol. 106, no. 5, pp. 755-762, 2017.

[67] R. Schanler, C. Lau, N. Hurst and E. O'Brian Smith, "andomized Trial of Donor Human Milk Versus Preterm Formula as Substitutes for Mothers' Own Milk in the Feeding of Extremely Premature Infants," *Pediatrics,* vol. 116, no. 2, pp. 400-406, 2005.

[68] D. Roberts, J. Brown, N. Medley and S. Dalziel, "Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth (Review)," *Cochrane Database Syst Rev,* vol. 3, p. CD004454, 2017.

[69] C. Crump, J. Sundquist, M. Winkleby and K. Sundquist, "Gestational age at birth and mortality from infancy into mid-adulthood: a national cohort study," *Lancet Child Adolesc Health,* vol. 3, no. 6, pp. 408-417, 2019.

[70] L. Doyle and P. Anderson, "Long-term outcomes of bronchopulmonary dysplasia," *Semin Fetal Neonatal Med,* vol. 14, no. 6, pp. 391-395, 2009.

[71] D. El Mazloum, L. Moschino, L. Bozzetto and E. Baradi, "Chronic Lung Disease of Prematurity: Long-Term Respiratory Outcome," *Neonatology,* vol. 105, no. 4, pp. 352-356, 2014.

[72] E. Priante, L. Moschino, V. Mardegan, P. Manzoni, S. Salvadori and E. Baraldi, "Respiratory Outcome after Preterm Borth: A Long and Difficult Journey," *Am J Perinatol,* vol. 33, pp. 1040-1042, 2016.

[73] S. Carraro, M. Filippone, L. Da Dalt, V. Ferraro, M. Maretti, S. Bressan, D. El Mazloum and E. Baraldi, "Bronchopulmonary Dysplasia: The earliest and perhaps the longest lasting obstructive lung disease in humans," *Early Hum Dev,* vol. 89, no. Suppl 3, pp. S3-S5, 2013.

[74] M. Vollsæter, O. Drange Røksund, G. Egil Eide, T. Markestad and T. Halvorsen, "Lung fuction after preterm birth: developmentfrom mid-childhood to adulthood," *Thorax,* vol. 68, pp. 767-776, 2013.

[75] L. Moschino, M. Stocchero, M. Filippone, S. Carraro and E. Baraldi, "Longitudinal Assessment of Lung Function in Survivors of Bronchopulmonary Dysplasia from Birth to Adulthood. The Padova BPD Study," *Am J Respir Crit Care Med,* vol. 198, no. 1, pp. 134-137, 2018.

[76] S. Kotecha, M. Edwards, W. Watkins, A. Henderson, S. Paranjothy, F. Dunstan and S. Kotecha, "Effect of preterm birth on later FEV1: a systematic review and meta-analysis," *Thorax,* vol. 68, pp. 760-766, 2013.

[77] S. Simpson, L. Turkovic, A. Wilson, M. Verheggen, K. Logie, J. Pillow and G. Hall, "Lung function trajectories throughout childhood in survivors of very preterm birth: a longitudinal cohort study," *LAncet Child Adolesc Health,* vol. 2, no. 5, pp. 350-359, 2018.

[78] J. Hurst, J. Beckmann, Y. Ni, C. Bolton, C. McEniery, J. Cockcroft and N. Marlow, "Respiratory and Cardiovascular Outcomes in Survivors of Extremely Preterm Birth at 19 Years," *Am J Respir Crit Care Med,* vol. 202, no. 3, pp. 422-432, 2020.

[79] L. Moschino, L. Bonadies and E. Baraldi, "Lung growth and pulmonary function after prematurity and bronchopulmonary dysplasia," *Pediatr Pulmonol,* vol. 56, no. 11, pp. 3499-3508, 2021.

[80] X. Gou, L. Yang, L. Pan and D. Xiao, "Association between bronchopulmonary dysplasia and cerebral palsy in children: a meta-analysis," *BMJ Open,* vol. 8, p. e020735, 2018.

[81] A. Bhandari and V. Bhandari, "Biomarkers in Bronchopulmonary Dysplasia," *Pediatr Respir Rev,* vol. 14, no. 3, pp. 173-179, 2013.

[82] C. Lal and N. Ambalavanan, "Cellular and humoral biomarkers of Bronchopulmonary Dysplasia," *Early Hum Dev,* vol. 105, pp. 35-39, 2017.

[83] F. Piersigilli, T. Lam, P. Vernocchi, A. Quagliarello, L. Putignani, Z. Aghai and V. Bhandari, "Identification of new biomarkers of bronchopulmonary dysplasia using metabolomics," *Metabolomics,* vol. 15, no. 2, p. 20, 2019.

[84] S. Carraro, G. Giordano, F. Reniero, G. Perilongo and E. Baraldi, "Metabolomics: A New Frontier for Research in Pediatrics," *J Pediatr,* vol. 154, pp. 638-644, 2009.

[85] F. Piersigilli and V. Bhandari, "Metabolomics of bronchopulmonary dysplasia," *Clin Chim Acta,* vol. 500, pp. 109-114, 2020.

[86] A. Schrimpe-Ritledge, S. Codreanu, S. Sherrod and J. McLean, "Untargeted metabolomics strategies – Challenges and Emerging Directions," *J Am Soc MAss Spectrom,* vol. 27, no. 12, pp. 1897-1905, 2016.

[87] "The Human Metabolome Database," [Online]. Available: https://hmdb.ca. [Accessed 15 March 2022].

[88] V. Fanos, M. Pintus, M. Lussu, L. Atzori, A. Noto, M. Stronati, H. Guimaraes, M. Marcialis, G. Rocha, C. Moretti, P. Papoff, S. Lacerenza, S. Puddu, M. Giuffrè, F. Serraino, M. Mussap and G. Corsello, "Urinary metabolomics of bronchopulmonary dysplasia (BPD): preliminary data at birth suggest it is a congenital disease," *J Matern Fetal Neonatal Med,* vol. 27(S2), pp. 39-45, 2014.

[89] M. Pintus, M. Lussu, A. Dessì, R. Pintus, A. Noto, V. Masile, M. Marcialis, M. Puddu, V. Fanos and L. Atzori, "Urinary 1H-NMR Metabolomics in the First Week of Life Can Anticipate BPD Diagnosis," *Oxid Med Cell Longev,* p. 7620671, 2018.

[90] E. Baraldi, G. Giordano, M. Stocchero, L. Moschino, P. Zaramella, M. Tran, S. Carraro, R. Romero and M. Gervasi, "Untargeted Metabolomic Analysis of Amniotic Fluid in the Prediction of Preterm Delivery and Bronchopulmonary Dysplasia," *PLoS ONE,* no. 11(10), p. e0164211, 2016.

[91] S. Carraro, G. Giordano, P. Pirillo, M. Maretti, F. Reniero, P. Cogo, G. Perilongo, M. Stocchero and E. Baraldi, "Airway Metabolic Anomalies in Adolescents with Bronchopulmonary Dysplasia: New Insights from the Metabolomic Approach," *J Pediatr ,* no. 166(2), pp. 234-9.e1, 2015.

[92] M. La Frano, J. Fahrmann, D. Grapov, T. Pedersen, J. Newman, O. Fiehn, M. Underwood, K. Mestan, R. Steinhorn and S. Wedgwood, "Umbilical cord blood metabolomics reveal distinct signatures of dyslipidemia prior to bronchopulmonary dysplasia and pulmonary hypertension," *Am J Physiol Lung Cell Mol Physiol,* no. 315(5), pp. L870-L881, 2018.

[93] C. Lal, J. kandasamy, K. Dolma, M. Ramani, R. Kumar, L. Wilson, Z. Aghai, S. Barnes, J. Blalock, A. Gaggar, V. Bhandari and N. Ambalavanan, "Early airway microbial metagenomic and metabolomic signatures are associated with development of severe bronchopulmonary dysplasia," *Am J Physiol Lung Cell Mol Physiol,* no. 315(5), pp. L810-L815, 2018.

[94] I. Jolliffe, Principal Component Analysis. Springer Series in Statistics., New York: Springer-Verlag, 2002.

[95] L. Breiman, "RandomForest," *Mach Learn,* vol. 45, pp. 5-32, 2001.

[96] M. Stocchero, M. De Nardi and B. Scarpa, "PLS for classification," *Chemometr Intell Lab Syst,* vol. 216, p. 104374, 2021.

[97] M. Stocchero, S. Riccadonna and P. Franceschi, "Projection to latent structures with orthogonal contraints for metabolomics data," *J Chemometr,* vol. 32, p. e2987, 2018.

[98] L. Sumner, A. Amberg, D. Barrett, M. Beale, R. Beger, C. Daykin, T. Fan, O. Fiehn, R. Goodacre, J. Griffin, T. Hankemeier, N. Hardy, J. Harnly, R. Higashi, J. Kopka, A. Lane, J. Lindon, P. Marriott, A. Nicholls, M. Reily, J. Thaden and M. Viant, "Proposed minimum reporting standards for chemical analysis. Chemical Analysis Working Group (CAWG). Metabolomics Standards Initiative (MSI)," *Metabolomics,* vol. 3, no. 3, pp. 211-221, 2007.

[99] "PubChem - National Center for Biotechnology Information," National Library of Medicine, [Online]. Available: https://pubchem.ncbi.nim.nih.gov. [Accessed 28 May 2022].

[100] M. Dambrova, C. Zuubier, V. Borutaite, E. Liepinsh and M. Makrecke-Kuka, "Energy substrate metabolism and mitochondrial oxidative stress in cardiac ischemia/reperfusion injury," *Free Radic BIo Med,* vol. 165, pp. 24-37, 2021.

[101] E. Makarova, M. Makrecka-Kuka, K. Vilks, K. Volska, E. Sevostjanovs, S. Grinberga, O. Zarkova-Malkova, M. Dambrova and E. Liepinsh, "Decreases in Circulating Concentrations of Long-Chain Acylcarnitines and Free Fatty Acids During the Glucose Tolerance Test Represent Tissue-Specific Insulin Sensitivity," *Front Endocrinol,* vol. 10, p. 870, 2019.

[102] P.-C. Teng, Y. Liang, A. Yarmishyn, Y.-J. Hsiao, T.-Y. Lin, T.-W. Lin, Y.-C. Teng, Y.-P. Yang, M.-L. Wang, C.-S. Chien, Y.-H. Luo, Y.-M. Chen, P.-K. Hsu, S.-H. Chiou and Y. Chien, "RNA Modification and Epigenetics in Modulation of Lung Cancer and pulmonary Disesases," *Int J Mol Sci,* vol. 22, p. 10592, 2021.

[103] T. Muller, M. Grimm, R. De Vieira, S. Cicko, T. Durk, S. Sorichter, G. Zissel and M. Idzko, "Local administration of uridine supresses the cardinal features of asthmatic airway inflammation," *Clin Exp Allergy,* vol. 40, no. 10, pp. 1552-1560, 2010.

[104] Y. Luo, H. Chen, R. Huang, Q. Wu, Y. Li and Y. He, "Guanosine and uridine alleviate airway inflammation via inhibition of the MAPK and NF-kB signals in OVA-induced asthmatic mice," *Pulm Pharmacol Ther,* vol. 69, p. 102049, 2021.

[105] S. Cicko, M. Grimm, K. Ayata, J. Beckert, A. Meyer, M. Hossfeld, G. Zissel, M. Idzko and T. Muller, "Uridine supplementation exerts anti-inflammatory and anti-fibrotic effects in an animal model of pulmonary fibrosis," *Respir Res,* vol. 16, no. 1, p. 105, 2015.

[106] E. Rozova, I. Mankovskaya, N. Belosludtseva, N. Khmil and G. Mironova, "Uridine as a protector against hypoxia-induced lung injury," *Sci Rep,* vol. 9, no. 1, p. 9418, 2019.

[107] M. Ernst, S. Rogers, U. Lausten-Thomsen, A. Bjorkbom, S. Svane Laursen, J. Courraud, A. Borglum, M. Nordentoft, T. Werge, P. Bo Mortensen, D. Hougaard and A. Cohen, "Gestational age-dependent development of the neonatal metabolome," *Pediatr Res,* vol. 89, no. 6, pp. 1396-1404, 2021.

[108] M. Caterino, M. Ruoppolo, M. Costanzo, L. Albano, D. Crisci, G. Sotgiu, L. Saderi, A. Montella, F. Franconi and I. Campesi, "Sex Affects Human Premature Neonates' Blood Metabolome According to Gestational Age, Parental Nutrition, and Caffeine Treatment," *Metabolites,* vol. 11, no. 3, p. 158, 2021.

[109] S. Stabler, R. Morton, S. Winski, R. Allen and C. White, "Effects of parental cysteine and glutathione feeding in a baboon model of severe prematurity," *Am J Clin Nutr,* vol. 72, no. 6, pp. 1548-1557, 2000.

[110] R. Kreider and J. Stout, "Creatine in Health nd Disease," *Nutriens,* vol. 13, p. 447, 2021.

[111] Y.-P. Shen, L. Fong, Z.-B. Yan and J.-Z. Liu, "Combining directed evolution of pathway enzymes and dynamic pathway regulation using a quorum-sensing circuit to improve the production of 4-hydroxyphenylaceti acid in Escherichia coli," *Biotechnol Biofuels,* vol. 12, p. 94, 2019.

[112] Z. Liu, R. Xi, Z. Zhang, W. Li, Y. Liu, F. Jin and X. Wang, "4-Hydroxyphenylacetic Acid Attenuated Inflammation and edema via Supressing HIF-1a in Seawater Aspiration-Induced Lung Injury in Rats," *Int J Mol Sci,* vol. 15, pp. 12861-12884, 2014.

[113] *DGR 2013/1308. LR finanziaria per l'esercizio 2008, n.1 del 27/02/2008, art 92 "Prevenzione, cura e riabilitazione delle malattie metaboliche". Approvazione del "Progetto di esecuzione dello screening neonatale allargato - Modello organizzativo",* Veneto, 2013.

[114] C. Stanley, D. Hale, G. Berry, S. Deleeuw, J. Boxer and J. Bonnefont, "Brief report: a deficiency of carnitine-acylcarnitine translocse in the inner mitochondrial membrane," *N Engl J Med,* vol. 237, no. 1, pp. 19-23, 1992.

[115] F. Andrade, J. Rodriguez-Soriano, J. Prieto, J. Elorz, M. Aguirre, G. Ariceta, S. Martin, P. Sanjurjo and L. Aldamiz-Echevarria, "The arginine-creatine pathway is disturbed in children and adolecents with renal transplants," *Pediatr Res,* vol. 64, no. 2, pp. 218-222, 2008.