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Review

Metabolomics of bronchopulmonary dysplasia

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ABSTRACT

The pathogenesis of Bronchopulmonary dysplasia (BPD) remains poorly understood. It is a multifactorial disease having a genetic-environmental basis. Although attempts have been made to identify a biomarker, none have been validated for clinical use to date. Metabolomics is a promising field of the "omics technologies" that could allow for better understanding of the pathogenesis and underlying mechanisms of BPD as well as facilitate detection of biomarkers. Identification of these biomarkers would improve diagnosis, identify patients in early disease stages and potentially target intervention. This review focuses on the evidence arising from metabolomics and its interaction with microbiomics and pharmacology with respect to pathogenesis and treatment options for this multifactorial complex disease.

1. Introduction

Bronchopulmonary dysplasia (BPD), a disorder arising from genetic-environmental risk factors, is one of the major diseases that still negatively affects premature infant prognosis. Although neonates are commonly exposed to the same risk factors (infections, hyperoxia, barotrauma), not all develop BPD [1]. As such, it is crucial to identify neonates predisposed to BPD. Unfortunately, there is still no specific treatment for BPD and the only way to modify severity and/or improve prognosis of this complex disease is prevention or early intervention.

Because the pathophysiology of BPD involves different molecular pathways, attempts have been made to identify biomarkers allowing early recognition of babies at risk for developing this disease [2]. The objectives of identifying a biomarker range from detection of a disease at earlier stages to better understanding of the progression of the disease, and finally developing suitable interventions in patient care and treatment to ameliorate the condition.

Several biomarkers have been identified until now, but none have been universally recognized as a reliable prognostic biomarker. In fact, due to the complexity of the pathogenesis, only one biomarker is probably not enough, and it would be necessary to develop a panel of biomarkers to allow us to identify those neonates that are prone to develop BPD.

Biomarkers can be identified in different biological liquids, as for example maternal blood, cord blood, peripheral blood, urine or in the tracheal aspirate (TA) or bronchoalveolar lavage fluid (BALF). Ideally, the most specific biomarkers should be identified in the TA/BALF, as these reflect more accurately what is going on in the lungs, whereas specimens from other parts of the body could be easily influenced by on-going inflammatory states in different organ systems.

Metabolomics is an "omic" science of endogenous metabolites as the final product of specific biochemical reactions occurring in the body as a result of the host response to a pathophysiological state.

This review will focus on the new evidence from metabolic studies conducted in human neonates to identify new biomarkers of BPD. The study of specific biochemical profiles of the organism's response during the clinical evolution of BPD can improve the timing, sensitivity and specificity of diagnosis and potentially improve treatment strategies.

2. Metabolomics

Metabolomics consists of the quantitative analysis of many low molecular mass metabolites (< 2000 Da) found in a specific cell, organ or organism, involving substrates or products of a defined metabolic pathway. The changes in metabolites composition reflects the interaction between a specific pathophysiological state, genetic predisposition,

Abbreviations: BPD, bronchopulmonary dysplasia; TA, tracheal aspirate; RDS, Respiratory Distress Syndrome; NMR, Nuclear Magnetic Resonance; MS, Mass Spectrometry; LC, Liquid Chromatography; GS, Gas Chromatography

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and environmental stimuli. Metabolites measured can be any chemical with a molecular weight < 2000 Da, for example peptides, lipids, organic acids, vitamins, minerals, drugs, amino acids, nucleic acids, carbohydrates, fatty acids, hormones, or drugs.

The metabolome is the complete collection of the metabolites found in the human body (22). The Human Metabolome Data Base (HMDB) is a freely available electronic database (http://www.hmdb.ca) for all the metabolites that have been identified. In the first publication in 2007 there was a listing of about 2500 metabolites [3]. The subsequent version in 2013 was expanded and enhanced, with the number of metabolites being identified reaching 40,000 [4]. To date, the number of metabolites identified has reached 114,000 [5].

During a disease or a change in a pathophysiological state, biochemical pathways (glycolysis, Krebs' cycle), lipid or amino acid metabolism, the response to oxidative stress can change, resulting in a different composition in metabolites. Therefore, metabolomics can provide a unique fingerprint of an organism, allowing us to understand, based on the composition of the metabolome, if those pathophysiological changes that predict the predisposition to the disease, have been triggered in that organism.

Two techniques are currently used to perform metabolic studies: the targeted and the non-targeted approach. The first approach consists of investigating some pre-known and expected metabolites. This technique can be only used when the pathophysiological state of a disease is already well known or at least partially understood. The non-targeted approach conversely consists of investigating all metabolites detectable in a sample to capture as much information as possible, providing a functional fingerprint of the pathological state that is being investigated. The metabolic pattern has to be investigated in different experimental groups to define a particular fingerprint related to a pathological process [6].

Metabolites can be identified and quantified using different technologies including nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Most MS methods must be coupled to various forms of liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis to facilitate compound separation. Each method is typically able to identify or characterize 50–5000 different metabolites or metabolite "features" at a time, but currently it is not possible to analyze the entire range of metabolites by a single analytical method [6].

Metabolomic studies require a comparison between normal patients and patients with the disease we are investigating, to be able to detect a difference between healthy and sick neonates. Authors have used metabolomics to investigate the relationship of metabolic pathways and the development of respiratory diseases such as asthma, pneumonia and bronchiolitis [7–9].

3. Metabolomics in respiratory distress syndrome (RDS)

To date, a few metabolomics studies in neonatology have focused on developing and validating the technique. The collection of metabolites in the first days of postnatal life and the comparison between neonates who will later develop the disease and neonates who will not, allowed investigators to highlight that preterm neonates predisposed to the development of BPD have, already from the first hours of life, a distinct response to oxidative stress and an altered metabolism, especially as regards to lipids and amino acids. RDS is a common antecedent in preterm neonates that predispose an infant to developing BPD.

Fabiano et al. were the first to conduct a preliminary study on metabolomics performed on the bronchoalveolar lavage fluid (BALF) of preterm neonates complicated by RDS [10]. The authors analyzed by NMR and GC–MS twelve BALF samples collected at birth prior to surfactant administration, post-surfactant administration during mechanical ventilation and at extubation. Some metabolites (undecane, decanoic acid, dodecanoic acid, hexadecanoic acid, octadecanoic acid, hexadecanoic acid methyl ester, 9-octadecanoic acid, tetracosanoic acid, myristic acid, phosphate) were over expressed in the BALF collected during mechanical ventilation after surfactant administration compared to BALF collected prior to surfactant administration. There were also several metabolites with an unknown structure, which the authors were not able to identify.

4. Metabolomics of BPD

4.1. Metabolites related to an alteration of oxidative stress

Fanos et al. studied the urinary metabolic profile at birth in 36 newborns with a gestational age below 29 weeks and birth weight below 1500 g. Neonates were subsequently divided in two groups: 18 neonates developed BPD and 18 neonates did not [11]. The authors found five discriminant metabolites in urine that allowed discerning neonates with and without BPD: lactate, taurine, and trimethylamine N-oxide (TMAO) were present only in neonates who developed BPD, myoinositol was increased in BPD patients, and gluconate was decreased in BPD patients. The increase in urinary lactate in the BPD patients is probably related to a shift to anaerobic respiration in response to hypoxia. On the other hand, taurine and TMAO have a fundamental biological role for membrane stabilization. Besides, taurine also plays important roles in calcium homeostasis, apoptosis, and detoxification.

Baraldi et al., on the other hand, used amniotic fluid to perform a metabolomics study to investigate the risk of spontaneous preterm delivery and BPD development in the offspring [12]. They performed an untargeted metabolic analysis using MS combined with ultra-performance LC on 32 amniotic fluid samples obtained from mothers who had undergone an amniocentesis between 21 and 32 weeks gestational age [12]. To determine if the amniotic fluid analysis could discriminate infants prone to develop BPD, they specifically studied 21 neonates, of whom 10 developed BPD and 11 did not. In the group with BPD there were higher levels of leucinic acid, hydroxy fatty acids and oxy fatty acids and a reduced level of S-adenosyl methionine, which is a methyl donor for biochemical methylation reactions and a precursor of glutathione, a powerful antioxidant of free radicals and other oxygen reactive species. In fact, a reduction of this metabolite has been associated with increased oxidative stress. The group without BPD was characterized by higher levels of 3β,16α-Dihydroxyandrostenone sulfate (a metabolite ascribable to Dehydroepiandrosterone sulfate or DHEAS). This finding confirms previous studies demonstrating an association between adrenocortical insufficiency (with reduced cortisol and DHEAS) and BPD development. The authors also performed a receiver operating characteristic curve (ROC) analysis for predicted outcomes (0.71, mean specificity = 0.78, mean sensitivity = 0.79). This study suggests that neonates exposed to higher levels of *in-utero* oxidative stress are the most likely to develop BPD, leading to a metabolic dysregulation identifiable already in the last few weeks of gestation.

Pintus et al. studied 18 neonates, of whom 7 developed BPD. A sample of urine was collected at 7 days of life and analyzed through ^{1}H NMR [13]. The authors found a different metabolic pattern between the two groups (neonates who developed BPD and neonates who did not) and the discriminant metabolites they identified were alanine, betaine, TMAO, lactate, and glycine. The urinary levels of glycine were lower in neonates developing BPD. Glycine has an important role in glutathione synthesis, which has an important antioxidant role, as previously described. Therefore, the observed decrease in glycine might be linked to an increase in oxidative stress or a lack of antioxidant defense.

All together, these studies confirm that oxidative stress [14], which is a known risk factor for BPD development, can start very early in life and/or even prenatally. It is therefore crucial that clinicians avoid any further oxidative injury to the most predisposed preterm infants.

Table 1
Metabolites involved in the pathogenesis of BPD.

	Patients predisposed to BPD	Patients not predisposed to BPD
Amniotic fluid [12]	⇧ leucinic acid ⇧ hydroxy fatty acids ⇧ oxy fatty acids ⇩ S-adenosyl methionine	⇧ 3β,16α-Dihydroxyandrostenone sulfate
Tracheal Urine [11]	⇧ lactate, taurine, TMAO ⇧ myoinositol ⇩ gluconate	
Tracheal aspirate [32]	⇩ acetyl-CoA/propionyl-CoA carboxylase	
BALF [20]	⇧ alanine, serine ⇧ citrulline ⇧ taurine	
Exhaled breath condensate [19]	⇧ lyso-phosphatidylcholine ⇧ platelet activating factor	⇧ phosphatidylserine
Umbilical cord blood [16]	⇩ phosphatidylcholines ⇩ sphingomyelins ⇧ choline	
Urine [13]	⇧ alanine, betaine ⇩ TMAO, lactate ⇩ glycine	

For abbreviations, please see text.

4.2. Metabolites related to an alteration of lipid metabolism

Pulmonary surfactant is a surface-active lipoprotein complex formed by type II alveolar cells. The proteins (10%) and lipids (90%) that make up surfactant have both hydrophilic and hydrophobic regions. The lipid component reduces surface tension. The two surfactant proteins (SP)-A and SP-D have an antibacterial role, while proteins SP-B and SP-C have a role in the production of lamellar bodies and in decreasing alveolar surface tension [15]. The main lipid component of surfactant is the phospholipid dipalmitoylphosphatidylcholine (DPPC) followed by other phospholipids (40%) such as phosphatidylcholine (PC) and phosphatidylglycerol and finally by cholesterol (10%).

Some authors have demonstrated that lipid metabolism is altered in neonates predisposed to the development of BPD. This could generate an alteration in surfactant composition in preterm neonates developing BPD. In fact, the altered composition of the lipid profile can be

evidenced even before birth in those neonates who will later develop BPD. La Frano et al. performed a study on umbilical cord blood metabolomics [16]. They used both a targeted and untargeted approach to analyze samples of umbilical cord blood from 10 neonates who later in life developed BPD compared to 10 controls. The main finding was a reduction in choline containing phospholipids (PC and sphingomyelins (SM)) and a rise in choline, showing that an alteration in lipid metabolism is responsible for an immaturity of lipid biosynthesis. In fact it has been demonstrated that the late maturation of PC biosynthesis is a contributor to the development of BPD [17]. Furthermore, the administration of a precursor of PC proved to be effective in ameliorating hyperoxia induced lung injury in an experimental rat model of BPD [18].

The dysregulation in lipid metabolites profile in neonates with BPD could be due to an altered surfactant composition, which may persist even beyond the neonatal period. Carraro et al. studied the metabolic profile of the exhaled breath condensate of a group of 20 adolescents with BPD compared to 15 healthy controls [19]. The samples were analyzed using a metabolomic approach based on MS, allowing identification of an altered lipid profile in the group of adolescents with BPD; the biomarkers identified were lyso-phosphatidylcholine, platelet activating factor (PAF), unsaturated phosphatidylcholine and a plasmenyl-phosphatidylserine. The authors attributed this different lipid composition to an altered surfactant composition, suggesting that the lungs of adolescents with BPD are characterized by long-term metabolic abnormalities. The dysfunction of surfactant that persist even after the first years of life could contribute to the persistent airflow limitation found in adolescent survivors of BPD.

In conclusion, the alteration and/or reduction of the lipid profile and especially that of lyso-, and unsaturated PC could be promising early markers of development of BPD and putative therapeutic targets for preventing or treating early BPD.

4.3. Metabolites related to an alteration of amino acid metabolism

Amino acid metabolism is another pathway that appears to be altered in neonates predisposed to the development of BPD.

In their study, Pintus et al. identified alanine, betaine, TMAO, lactate, and glycine as discriminant metabolites between patients with BPD and controls [13].

Alanine is a nonessential amino acid, as it can be synthesized from pyruvate and branched chain amino acids such as valine, leucine, and isoleucine. It has close links to metabolic pathways such as glycolysis, gluconeogenesis, and the citric acid cycle. The synthesis of alanine is directly proportional to the concentration of pyruvate that mainly increases when there is a high rate of degradation of fatty acids for energy purposes and is converted, under anaerobic conditions, both into alanine and lactic acid. Furthermore, the glucose-alanine cycle is stimulated by the increase of plasmatic levels of glucocorticoids (cortisol), in response to stressful events.

In a study performed on the BALF of 68 preterm neonates collected in the first day of life [20], we demonstrated that neonates who subsequently develop BPD (44 neonates) have higher levels of alanine, serine, taurine and citrulline than neonates who do not develop BPD (24 neonates). Taurine, which has a fundamental biological role for osmoregulation, membrane stabilization, and detoxification, appears to be increased in relation to hypoxia. Similarly, Fanos et al found an increased level of taurine in neonates with BPD respect to controls [11]. Citrulline is a non-essential amino acid and it is the precursor of arginine, which is a substrate for the production of nitric oxide (NO).

The identification of altered amino acids in neonates who later develop BPD may allow us to better understand which metabolic pathways are activated precociously in newborns with BPD.

Table 1 summarizes the metabolites that appear to be involved in the pathogenesis of BPD.

4.4. Pharmacometabolomics of BPD

Pharmacometabolomics is a novel field of metabolomics aimed at identifying variability in drug response. The variations in the metabolome before and after treatment with a specific drug can elucidate the mechanisms of variation in drug response between patients and may help establish treatment outcome. Furthermore, if a metabolic signature of non-responders was available before treatment, then infants in the non-responder group could be spared that specific drug and could receive alternate therapies.

In their paper Lewis et al. [21] performed a prospective cohort steroid pharmacometabolomic study in preterm infants with evolving or established BPD, with the purpose of identifying metabolomic changes that correlate with drug response and drug failure. They enrolled 10 preterm infants less than 32 weeks gestation treated with systemic dexamethasone as per their clinical protocol. Blood and urine samples were collected 24 h prior to treatment and once after 6 days. They performed an untargeted metabolomics analysis and found that 11 metabolites in blood and 15 in urine were significantly different between pre-steroid and post-steroid treatment. Gluconic acid and caprylic acid were the most significantly modified metabolites in serum samples whereas isohexanoic acid and sucrose were the most modified metabolites in urine samples. Before treatment serum baseline xylitol level and urinary baseline saccharic acid were the most associated with the degree of steroid response. During treatment urine gluconic acid lactone, uridine, and mannitol were found to correlate with the degree of steroid response. The more the metabolites decreased with treatment, the better the clinical response. In conclusion, the authors showed that steroid treatment leads to a change in serum and urinary metabolites that are mostly involved in the inflammatory cascade and oxidative stress.

Based on these preliminary findings pharmacometabolomics seems a promising field to better identify which patients seem to be responders to a specific treatment in order to avoid ineffective treatments that expose children to side effects without the benefits.

4.5. Interaction with microbiomics

The term microbiota defines the community of microorganisms (commensal, symbiotic and pathogenic microorganisms) that typically inhabit the body or an organ whereas the microbiome represents the collective genomes of the microbial community that colonizes the body. The human body contains over 10 times more microbial cells than human cells and the whole bacterial genome contains at least 100 times as many genes than our own genome. Microbiota have a crucial role for immunologic, hormonal and metabolic homeostasis of their host.

As most of these bacteria cannot be cultured, the whole microbiome could be detected thanks to high-throughput technologies allowing discovery of the presence of bacteria thanks to the identification of its gene. The Human Microbiome Project [22], launched in 2008 by the National Institute of Health, has precisely the goal to characterize the bacteria that colonize a part of the body and to determine whether there are associations between changes in the microbiome and the onset of diseases.

In preterm infants, many microbiome studies have focused on the gut and on the development of necrotizing enterocolitis [23,24]. Clinicians have always considered the lower respiratory tract of healthy individuals as sterile, but recent evidence has shown that even healthy lungs have a colonizing microbial community. Fetal lungs have always presumed to be sterile so that lungs acquire their microbial communities in the immediate post-delivery period depending on the mode of delivery: vaginally delivered infants acquire bacterial communities resembling the microbial vaginal environment (*Lactobacillus, Prevotella*) and C-section infants acquire mostly skin microbiota (*Staphylococcus, Corynebacterium, Propionibacterium*) [25]. Nevertheless, some studies have demonstrated that the lung of preterm infants is colonized already

at birth, suggesting that the amniotic environment is not sterile [26]. Bacterial infection is a well-known inflammatory trigger and as inflammation can predispose to BPD [27], it is easily understandable that the presence of some pathogenic bacteria in the lungs can be associated with the development to BPD. Specifically, for example, *Ureaplasma urealyticum* infection has been repeatedly associated with BPD [28].

Nevertheless, even just a dysbiosis can be associated with an increased risk of developing BPD. Several studies have demonstrated that neonates predisposed to BPD already at birth have a composition of colonizing bacteria that is decreased in *Lactobacillus*, that have strong anti-inflammatory properties [29]. Furthermore, neonates who develop BPD have also a reduced diversity of the lung microbiome [30]. Pammi et al. performed a systematic review of six studies that relate the lung microbiome with BPD development [31]. Most studies reported an increased microbial community turnover, a change in the relative abundance of *Proteobacteria* and *Firmicutes*, and a decrease in *Lactobacilli* in patients predisposed to BPD. Similarly Lal et al. studied the airway microbiome at birth in 23 preterm infants (10 predisposed to BPD and 13 resistant) and they found that the microbiome of infants after diagnosis of BPD was characterized by increased phylum Proteobacteria (among which *Enterobacteriaceae* were more represented) and decreased phyla Firmicutes and Fusobacteria [32]. Contrariwise, *Lactobacilli* were less abundant at birth in preterm neonates predisposed to BPD and in neonates born to mothers with chorioamnionitis.

It is still not clear which mechanism leads to a different predisposition to BPD based on the different microbiome. *Lactobacilli* are known to have strong anti-inflammatory properties [27] and the described dysbiosis may contribute to the airway inflammation associated with BPD. Metabolomics can help us understand the metabolic pathways that are activated in newborns who develop BPD based on the microbiological pattern they have in their airways/lungs.

Lal et al. investigated the relationship between airway microbiome and metabolome at birth [33]. The authors collected TA samples at 6 h of life from infants with a gestational age < 28 weeks. Fifteen neonates were predisposed to develop BPD and 15 were not. In BPD patients, they found an increase in *Proteobacteria* abundance, a reduction in *Lactobacilli*, and a decrease in the ratio acetyl-CoA/propionyl-CoA carboxylase, indicating a reduced fatty acid β-oxidation pathway [33]. The changes related to lipid metabolism pathways suggest that the metabolic activity of the airway microbiome may modulate the metabolome. The bacterial lipid metabolites may in turn increase airway inflammation leading to BPD.

Even intestinal microbiota can modulate the microbiome and the predisposition to BPD. In the aforementioned study, Pintus et al. [13] demonstrated in the urine samples of patients predisposed to BPD there was an increased concentration of betaine and a reduction of TMAO. The intestinal microbiota has an obligatory and pivotal role in the formation of TMAO [34]. The decreased levels of TMAO in the BPD predisposed patients suggest an alteration of the intestinal microbiota.

Hence, the identification of a specific microbiome contributing to the change of the metabolome predisposing to BPD could represent a target for antibiotic therapies to lower the incidence of BPD.

5. Conclusions

Until now, it has not been possible to identify a single protein biomarker for a complex disease as BPD. A panel of biomarkers derived from the interaction of genomics, microbiomics, and metabolomics could be promising.

Specifically, utilizing metabolomics approaches may be crucial in order to identify which neonates should be treated precociously in order to decrease the risk of developing the disease. Metabolomic information correlating with the long-term outcomes of BPD would be useful. Pharmacometabolomics seems a promising field to better identify which patients can benefit from targeted drug therapy. Furthermore, the abnormal metabolic pathways identified could represent the target of novel future therapies.

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