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Genomics, microbiomics, proteomics, and metabolomics in bronchopulmonary dysplasia

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Abstract

Bronchopulmonary Dysplasia (BPD) is a disorder with a multifactorial etiology and highly variable clinical phenotype. Several traditional biomarkers have been identified, but due to the complex disease phenotype, these biomarkers have low predictive accuracy for BPD. In recent years, newer technologies have facilitated the in-depth and unbiased analysis of 'big data' in delineating the diagnosis, pathogenesis, and mechanisms of diseases. Novel systems-biology based 'omic' approaches, including but not limited to genomics, microbiomics, proteomics, and metabolomics may help define the multiple cellular and humoral interactions that regulate normal as well as abnormal lung development and response to injury that are the hallmarks of BPD.

Keywords

BPD; Genetics; Metabolome; Proteome; Microbiome; Systems biology

Bronchopulmonary Dysplasia (BPD) has a clinical operational definition based on oxygen receipt at a specific postnatal time point (usually 36 weeks' postmenstrual age or PMA), with limited correlation with underlying disease phenotype.[1] Although the operational definition of BPD has evolved over the past half-century,[2–4] it does not describe the underlying structural or molecular cardio-pulmonary pathophysiology. For example, severe BPD markedly differs from that of mild or moderate BPD, both in terms of clinical features as well as in terms of genetic predisposition and is not just on the extreme end of the continuum of the spectrum of the clinical operational definition.[5]

Newer technologies have facilitated the gathering of large data sets, often by high-throughput assays, with subsequent analyses of these data sets through bioinformatic methods. In these studies, the researcher obtains a very highly detailed dataset of the changes that occur in response to a defined perturbation, such as the induction of a normal or diseased state. These large-scale datasets on the genome, proteome, metabolome, microbiome etc. are often referred to as 'omic' data sets.[6] An advantage of these

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technologies is that they can be used with samples (blood, tracheal aspirates or TA, urine, etc.) that can be collected using minimally invasive or non-invasive methods, which enables serial sampling for longitudinal analyses of markers associated with normal or abnormal growth and development. The analytical approaches involved are not driven by any a *priori* hypotheses, thus permitting unbiased 'omic' pattern profiling of a given pathological condition to be identified, followed by recognition of potential targets in the disease profile, and eventually to formulate hypothesis for diagnostics or theranostics. A better understanding of the 'omic' data related to BPD may provide insight not only into the predisposition to BPD (e.g. from genomic data) but also into the pathogenesis of disease (e.g. by comparing gene expression, proteomic, or metabolomic data from TA, blood, or lung tissue from individuals with and without BPD), and prediction of therapeutic response (e.g. change in 'omic' biomarkers following initiation of specific therapies) (Fig. 1).

In this manuscript, we will discuss the various new 'omic' systems biology approaches that are currently available or may be available in the future for informing clinicians regarding the diagnosis, prognosis, and possible treatment of BPD. This review is an update on recent advances in the field, with a brief overview of the evidence already covered in our previous manuscripts.[1,7,8]

Genomics of BPD

The advanced understanding of the human genome sequence has led to large-scale generation of data sets and added a new dimension to biomedical research. The genetic factors that contribute to BPD susceptibility have been the subject of much investigation in recent years. The role of genetics in BPD was initially proposed by Parker et al.[9] who found a high concordance in 108 twin pairs of birth weights less than 1500 g. Familial and genetic susceptibility to BPD was evaluated in a multicenter retrospective study of twin pairs born at ≤32 weeks of gestation by Bhandari et al.[10] After controlling for the effects of covariates, the twin data showed that 65.2% (95% CI: 53–79%, $p<0.001$) of the variance in liability for BPD could be accounted for by genetic and shared environmental factors. The genetic component was estimated from the correlation between monozygotic twins beyond that of dizygotic twins, and the observed concordance in monozygotic twins was significantly higher than the expected concordance. After controlling for covariates, genetic factors were considered to account for as much as 53% (95% CI 16–89%, $p=0.004$) of the variance in liability for BPD.[10] Lavoie et al.[11] evaluated the heritability of BPD using clinical data from 318 twin pairs of known zygosity <30 weeks of gestation. Model-fitting analyses indicated that genetic effects accounted for 79% of the observed variance in moderate to severe BPD susceptibility.[11]

Genomic variants predisposing to BPD may be single nucleotide polymorphisms (SNPs), which may increase susceptibility to the disease. Candidate gene variants associated with BPD have been reviewed recently[12,13] and will not be discussed in detail in this manuscript.

Hadchouel et al.[14] conducted a genome wide analysis and identified SPOCK2 as a new possible candidate susceptibility gene for BPD, but this target was not confirmed in more recent studies by Wang et al.[15] or Ambalavanan et al.[5] The second of these studies was

an integrated genomic analysis was conducted by the Eunice Kennedy Shriver National Institutes of Child Health and Human Development Neonatal Research Network.[5] Using a DNA repository of extremely low birth weight infants, a genome-wide scan was conducted on 1.2 million genotyped SNPs, and an additional 7 million imputed SNPs followed by genome-wide association and gene set analysis for BPD or death, severe BPD or death, and severe BPD in survivors. Known pathways of lung development and repair (the cell-surface glycoprotein CD44, phosphorus oxygen lyase activity) and novel molecules and pathways (adenosine deaminase, targets of microRNA or miR-219) were found to be involved in the genetic predisposition to BPD.[5]

Other approaches utilizing gene expression profiling, such as those by Bhattacharya et al.[16] and Pietrzyk et al.[17] have also identified different pathways associated with the genetic origins of BPD.

While SNP array profiling has been traditionally used for genome wide analysis, far more in-depth analysis including analysis of rare variants is available with next-generation sequencing methods, such as whole exome sequencing (WES) and whole genome sequencing (WGS), the costs of which are rapidly decreasing. Carrera et al.[18] did whole exome sequencing to identify non-common variants in patients with BPD. The top candidate genes 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. Li et al.[19] have also performed exome sequencing on 50 BPD-affected and unaffected twin pairs using DNA isolated from neonatal blood spots and identified genes affected by extremely rare nonsynonymous mutations.

In recent years, RNA sequencing methods have been developed, which can evaluate protein-coding mRNA, long non-coding RNA (lncRNA), transcripts of pseudogenes, and miRs. A RNA sequencing study utilizing the rodent model of BPD was conducted by Bhattacharya et al.[20] in which expression patterns for selected genes were validated by qPCR followed by mechanistic testing. In this study, the canonical pathways dysregulated in hyperoxia included nuclear factor (erythryoid-derived-2)-like 2 (Nrf2)-mediated oxidative stress signaling, p53 signaling, endothelial (e)NOS signaling, and aryl hydrocarbon receptor (Ahr) pathways. Further cluster analysis identified Cyclin D (CCND1), cyclin dependent kinase inhibitor 1A (CDKN1A), and Ahr as critical regulatory nodes in the response to hyperoxia, with Ahr serving as the major effector node. Other investigators have used the hyperoxia-exposed Nrf2 null mutant newborn mice, along with transcriptomic analyses, to identify the effector molecules of lung injury.[21] In another study, Salaets et al.[22] conducted RNA sequencing of preterm rabbit lungs after seven days of hyperoxia exposure and identified changes in inflammatory, oxidative stress and lung developmental pathways. In a recent study utilizing human lung tissue, Kho et al.[23] analyzed whole-lung transcriptome profiles of 61 females and 78 males at 54–127 days post conception from nonsmoking mothers, using unsupervised principal component analysis and supervised linear regression models. This study, which utilized banked human fetal lung tissue found post conceptional age to be a more dominant factor than sex, in the effect of early fetal lung development on disease risk. A major limitation of this study was the inability to adjust for differences in race and potential confounders such as in utero exposures, due to limited availability of

phenotypic information. Further human studies utilizing RNA sequencing technologies are warranted in preterm cohorts.

In previous years, most research was focused on messenger RNA as the primary link between DNA sequence and its effects on protein synthesis and other aspects of cell behavior. In recent years, non-coding RNA including miRs, other small RNA, and long non-coding RNA (lncRNA) have been recognized as important mediators of normal growth, development, and disease. In a mouse model of BPD, investigators noted that 882 lncRNAs were upregulated, and 887 lncRNAs were downregulated in BPD lung tissues. Several miRs have been described to play a role in branching morphogenesis, a key step in early lung development.[24] The current evidence of the role of miRs in late lung development and BPD has been summarized by Nardiello and Morty recently.[24] We have highlighted the role of miR-489 in guiding alveolar septation by modulation of its target genes insulin-like growth factor (IGF-1) and tenascin-C (TNC).[25] In addition, we have recently identified airway exosomal miRs which are predictive of, and are involved in BPD pathogenesis.[26] Decreased airway exosomal miRs levels at birth (miR-876-3p, miR-378b, miR-20a-50 +miR-20b-5p, miR-1254 and miR-1252-5p) were highly predicted of severe BPD in extremely preterm infants.[26] Also, as mentioned previously, in the integrated genomic analyses conducted by the NICHD NRN, the pathway with lowest false discovery rate (FDR) for BPD/death was the targets of miR219. In another study, Syed et al.[27] recently showed that pharmacologic miR-34a inhibition may be a therapeutic option to prevent or ameliorate hyperoxia induced lung injury in neonates. The authors found that lung miR-34a levels are significantly increased in lungs of neonatal mice exposed to hyperoxia; deletion or inhibition of miR-34a improved the pulmonary phenotype and BPD-associated pulmonary arterial hypertension (PAH) in BPD mouse models. In addition, administration of angiopoietin-1, which is one of the downstream targets of miR-34a, was able to ameliorate the BPD pulmonary and PAH phenotypes.[27] In addition, utilizing 3 independent cohorts of human lung samples, the investigators reported a significant association of increased pulmonary miR-34a expression in neonates with respiratory distress syndrome (RDS) and BPD.[27]

Efforts are needed to develop sophisticated computational methods for the integration of miRs, microRNA targets, transcription factors and other important components of the transcriptome into biologically relevant networks, in the context of BPD.

Microbiomics

Human microbiome refers to the diverse microbial population that reside within or on various surfaces of the human body.[28] Around 10–100 trillion human microbial cells are harbored by each person[29] and human microbiome research has expanded significantly over the past few years. The Human Microbiome Project (HMP) was added to the National Institutes of Health (NIH) Roadmap for Medical Research in 2017 and since then, several human body sites including the nares, oral cavity, skin, gastrointestinal tract, and urogenital tract have been studied by the HMP.[29] Many studies have established that the microbiome influences the host immune system and plays a significant role in acquiring as well as preventing disease states. Many studies on the microbiome have so far focused mainly on the role of the gut microbiome.[30,31] The study of the airway microbiome in perspective with

pulmonary health is still an area of emerging field of research.[32] Until recently, evidence of pathogenic roles for specific alterations in airway microbiota was strongest in lung diseases such as cystic fibrosis, chronic obstructive pulmonary disease (COPD) and asthma, but it was not clear how the airway microbiome is initially established in newborn infants.[32,33]

Historically, the fetus and fetal lungs were considered sterile, but we recently discovered that the airways of infants at birth harbor a distinct microbial signature.[34] In this study, 16S sequencing microbiome analysis was conducted on TA obtained from preterm and term intubated babies at birth or shortly after, longitudinally, and after the diagnosis of severe BPD. The airways of infants with BPD showed increased abundance of *Proteobacteria* and decreased *Lactobacillus*. Serial longitudinal sampling in preterm infants who went on to develop severe BPD showed a temporal dysbiosis, with an increase in *Proteobacteria* and decrease in *Lactobacillus* on the way to development of severe BPD at 36 weeks' PMA. In addition, an early microbial dysbiosis, that of decreased *Lactobacillus* at birth, was predictive for the development of severe BPD. Genus *Lactobacillus* has been known to have strong anti-inflammatory properties[35–37] and has been shown to regulate alveolar development in animal models.[38] Hence, favorable bacteria inoculation as "respiratory probiotics" may be further studied as a potential therapeutic strategy for BPD and other pulmonary disorders. The early airway microbiome may prime the developing pulmonary immune system, thus setting the stage and predisposing to subsequent pulmonary disease. Studies by Lal et al. and Lohman et al. also compared extremely low birth weight (ELBW) infants with BPD and those without BPD.[34,39] Lohmann et al. reported decreased bacterial diversity as estimated by number of observed species and Shannon diversity index in babies who developed BPD compared to those who did not, based on the sample at intubation (first 24 h of life).[39] Our study reported differences in microbe diversity and abundance (alpha diversity and beta diversity) between ELBW infants and infants with established BPD and between full term (FT) infants and infants with established BPD. Other studies have compared airway microbiome against grades of severity of BPD, although no preterm infants without BPD were analyzed.[40,41] In the cross-sectional dataset of samples obtained from days 5 to 9, in preterm infants, the bacterial load, Shannon alpha diversity index and evenness were not significantly different across BPD groups. Lohmann et al.[39] suggested that reduced diversity of the microbiome may be an associated factor in the development of BPD. Abman et al.[41] demonstrated by 16S techniques in another study that included 10 infants that early bacterial colonization with diverse species is present in the airways of intubated preterm infants, and can be characterized by bacterial load and species diversity.[41] Lohmann et al.[39] also reported that microbial communities at the phylum level showed increase in relative abundances of Firmicutes and decrease in relative abundances of Proteobacteria over time in BPD group in contrast to the relatively diverse and stable community in the non-BPD group. No correlation was found between the levels of cytokines, lipoteichoic acid (LTA), and lipopolysaccharide (LPS) or variations in the community composition. In contrast, our study[34] reported that the airway microbiome of infants after diagnosis of BPD was characterized by increased phylum Proteobacteria and decreased phyla Firmicutes and Fusobacteria when compared to newborn FT infants matched for PMA. The reasons for this difference may due to differences in clinical characteristics of the included patients, methods or environmental ecology differences

between the units where the studies were conducted. *Gamma Proteobacteria* were more abundant in BPD infants whereas *Alpha Proteobacteria* were in lower abundance in BPD infants compared to newborn ELBW and FT infants. At the genus level, the most abundant *Proteobacteria* in BPD patients were *Enterobacteriaceae*. Genus *Lactobacillus* was statistically less abundant in the early airway microbiome of infants who later developed BPD both in the discovery and validation cohorts, but the overall abundance of Lactobacillus was small.[34] In the Wagner cohort, *Staphylococcus* (68%) and *Ureaplasma* (18%) dominated the microbiome but the relative abundance of these bacteria was not significantly different across BPD severity.[40] Wagner analyzed 233 samples from 94 preterm infants which revealed that preterm infants who eventually developed severe BPD exhibited greater bacterial community turnover with age, assessed by beta-diversity measures of Shannon beta diversity and Morisita-Horn pair wise comparison measures.[40] In this study, infants with more severe BPD acquired less *Staphylococcus* in the first days after birth and had higher initial relative abundance of *Ureaplasma*. Lal et al.[34] followed 5 preterm infants longitudinally and despite having multiple courses of antibiotics, these infants had a distinct temporal dysbiotic change with a decrease in *Firmicutes* and increase in abundance of *Proteobacteria* over time.

Despite these studies demonstrating associations between dysbiosis and BPD, the direction of causality between airway injury during development and respiratory colonization with microorganisms remains unsettled. Further mechanistic studies are necessary to determine the exact causal implication of dysbiosis in BPD. Data so far suggests that the early airway microbiome may prime the developing pulmonary immune system, and dysbiosis in its development may set the stage for subsequent lung disease, especially in extremely preterm infants. There is also need for further intervention trials targeting antibiotics, prebiotics and probiotics use, to identify if the airway microbiome can be intentionally manipulated to provide a therapeutic benefit.

Proteomics

Proteomics is the large scale study of the structure and function of proteins and yet only 4% of proteomic studies aimed at lung injury and disease have focused on pediatric lung disease.[42] Several individual proteins have been implicated in the pathogenesis of BPD, and have been described in detail in our previous publications,[1,43] although studies utilizing unbiased proteomic analysis for the disease are lacking. Proteomic analysis may help study the networks of proteins that provide real time status of disease state and modulation of protein function.[44] In the first such pulmonary study in preterm infants, Magagnotti et al.[45] conducted a proteomic analysis of TA from infants with BPD and controls, using mass spectroscopy. The results were further validated by western blotting. The authors found a clear differential expression in the proteome of the 23–25 weeks gestational age (GA) group and the 26–29 weeks GA group. Surfactant protein-A2 (↑), annexin-3 (↑), calcium and integrin binding protein-1 (↓), leukocyte elastase inhibitor (↓), chloride intracellular channel protein 1 (↓) and calcyphosine (↓) were differentially expressed in severe BPD patients with lower GA (23–25 weeks gestation). After adjusting for severity of disease, calcyphosine, calcium and integrin binding protein-1 and chloride intracellular channel protein 1, were found to differentiate between mild and severe BPD; however, annexin-3 was found to be

more related to development. A major limitation of this study was the small sample size and marked heterogeneity in the clinical phenotype of the patients. Analyzing TA fluid for proteomics analysis suffers from the limitation of defining an appropriate control. Serum sample analysis is considered low risk making it ideal for biomarker discovery studies; however, changes within serum are often very small and moreover, pinpointing changes specific to the lung can be difficult. Hence despite the limitations, TA fluids are probably the best option available to analyze local effects in preterm lungs of mechanically ventilated infants. As many preterm infants are managed with continuous positive airway pressure (CPAP) and/or oxygen supplementation, evaluation of TA in these infants is not possible. However, infants who do not require mechanical ventilation may generally be considered at lower risk of BPD or death. Urine proteomic analysis has been used to discover novel biomarkers for other diseases of prematurity such as necrotizing enterocolitis,[46] and similar studies utilizing urine proteomics are currently underway for BPD.

Metabolomics

Metabolomics is an emerging ‘omics’ science involving the comprehensive characterization of metabolites and metabolism in biological systems.[47] It includes analysis of low molecular weight metabolites created by cellular metabolic pathways through the use of mass spectrometry or nuclear magnetic resonance spectroscopy. Extremely preterm infants are very different from other patient populations from a developmental standpoint and may have a markedly different metabolic signature in different organ systems even at baseline. In addition, factors such as oxygen exposure, mechanical ventilation, diet, drugs, antibiotics, resident microbiome, etc., may further alter the metabolic profiles and hence effect disease pathogenesis. Recently, Baraldi et al.[48] performed metabolic profiling of amniotic fluid (AF) in women with symptoms of preterm labor to predict the risk of spontaneous preterm birth and BPD development in the offspring. Twenty-four of the 32 AF samples were obtained from trans-abdominal amniocentesis, the other 8 by amniocentesis at the time of cesarean delivery. In this study, the AF from mothers in the preterm delivery group with BPD showed higher levels of leucinic acid, hydroxy fatty acids (putative metabolite: 4-Hydroxy-3-methylbenzoic acid and 2-hydroxy caprylic acid), oxy fatty acids (putative metabolite: 3-oxo-dodecanoic acid), and a metabolite ascribable to a sulphated steroid. The group without BPD was characterized by higher levels of s-adenosylmethionine and amino acid chains and 3b,16a-dihydroxyandrostenone sulfate (DHEAS) compared to the BPD group. This study suggests that the onset of BPD may be associated with a perturbed AF metabolic pattern during intra-uterine life. Although the authors performed internal cross-validation, the absence of external validation and small sample size were major limitations of this study.

Organic volatile metabolites may also be detected by portable devices such as electronic noses.[49] In a recent study by Rogosch et al.[50] the smellprints of volatile organic compounds measured with an electronic nose (Cyranose 320; Smiths Detection Group Ltd, Watford, United Kingdom) differed between TA from preterm infants with or without subsequent development of BPD.[50] In a study of adolescents, Carraro et al.[51] found that the metabolomic analysis of exhaled breath condensates distinguishes children who have had BPD from healthy individuals, suggesting that metabolic abnormalities persist in the lung

of survivors of BPD, although the functional relevance of these changes is unclear. These studies emphasize the importance of conducting longitudinal studies to monitor changes in the airway metabolome among premature infants. Systems biology approaches identifying complex metabolomic dysregulation in the setting of multifactorial diseases such as BPD provide a unique opportunity to develop new diagnostic or therapeutic strategies as the metabolites may serve as a biomarker of, or mediate lung function.

Conclusion

More detailed data collection of clinical variables for improved disease phenotyping, in addition to careful determination of unbiased, specific, temporal, 'omic' biomarkers, are necessary for BPD management and treatment.[52,53], With the discovery of the presence and role of the neonatal airway microbiome in BPD,[34] the study of environmental pathogens and host responses are required, in addition to strategies evaluating genomic, microbiomic, proteomic, and metabolomics data.

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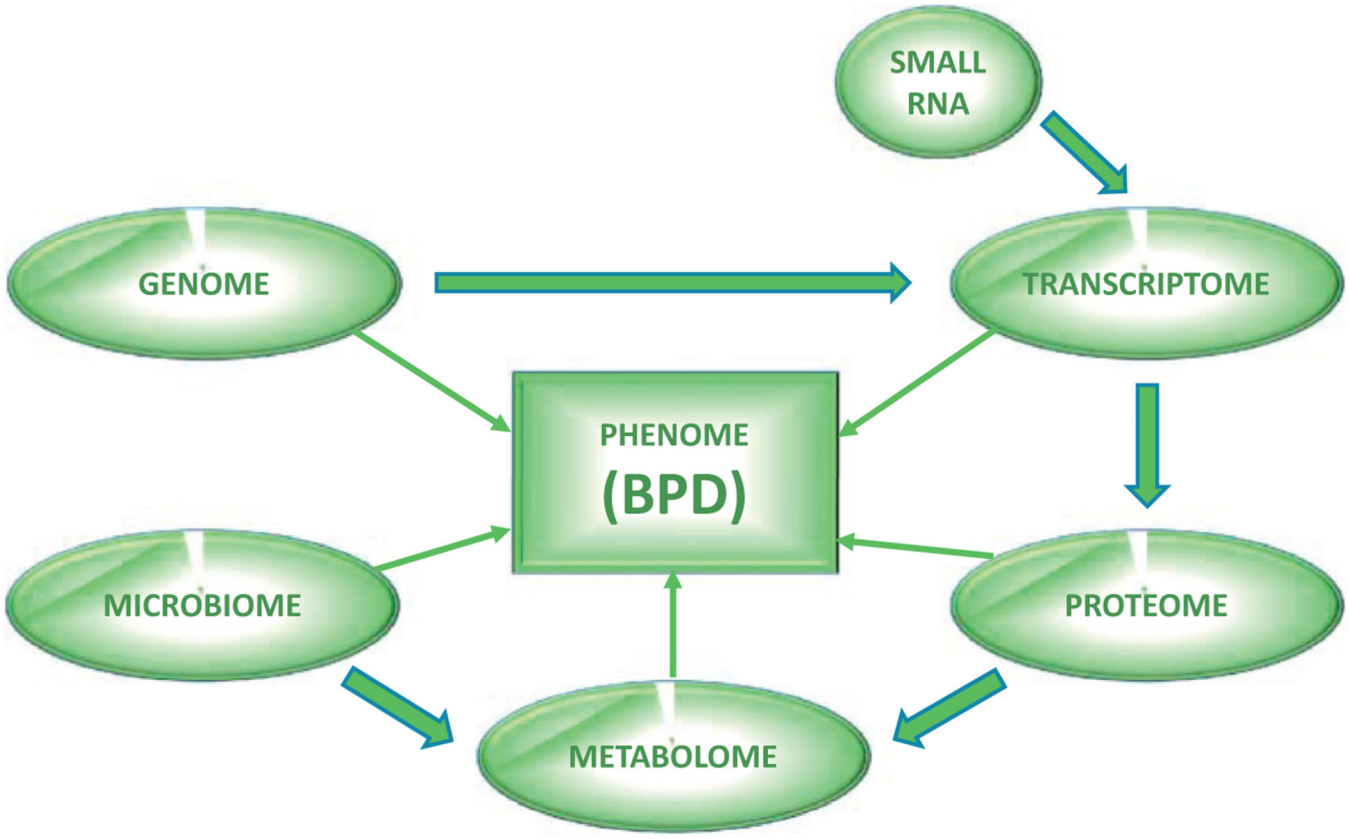

Fig. 1 –.

Interaction of the various “omic” profiles inform about and contribute to the pathogenesis of the target disease, bron-chopulmonary dysplasia or BPD.