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RESEARCH ARTICLE

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Multiomics endotyping of preterm infants with bronchopulmonary dysplasia and pulmonary hypertension—A pilot study

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Abstract

Pulmonary hypertension associated with bronchopulmonary dysplasia is a severe complication of preterm birth resulting in high mortality of up to 50% within the first 2 years of life. There is a direct relationship between bronchopulmonary dysplasia severity and incidence of associated pulmonary hypertension. However, it is challenging to clinically characterize severe bronchopulmonary dysplasia with and without pulmonary hypertension and there is need for better understanding of the two entities. Our main objective is to identify markers to help understand biological processes and characterize infants with pulmonary hypertension associated with bronchopulmonary dysplasia using tracheal aspirates. We conducted an unbiased multiomic analysis of tracheal aspirates via microRNA (miRNA) polymerase chain reaction arrays, RNA sequencing, and mass spectrometry proteomics in preterm infants with severe bronchopulmonary dysplasia with and without pulmonary hypertension ($n = 46$). Our pilot study analysis revealed 12 miRNAs (hsa-miR-29a, has-miR-542-3p, has-miR-624, has-miR-183, hsa-miR-501-3p, hsa-miR-101, hsa-miR-3131, hsa-miR-3683, hsa-miR-3193, hsa-miR-3672, hsa-miR-3128, and hsa-miR-1287), 6 transcripts (IL6, RPL35P5, HSD3B7, RNA5SP215, OR2A1-AS1, and RNVU1-19), and 5 proteins (CAPS, AAT, KRT5, SFTPB, and LGALS3BP) with significant differential expression in preterm infants with severe lung disease with pulmonary hypertension when compared with infants with severe lung disease but no pulmonary hypertension. Pathway analysis of the integrated multiomic expression signatures revealed NFkB, VEGF, SERPINA1, IL6, and ERK1/2 as target molecules and cellular development, cellular growth and proliferation, and cellular movement as key affected molecular functions. Our multiomic analysis of tracheal aspirates revealed a comprehensive thumbprint of

miRNAs, mRNAs, and proteins that could help endotype infants with severe lung disease and pulmonary hypertension.

KEYWORDS
bronchopulmonary dysplasia, multiomics, preterm infants, pulmonary hypertension, tracheal aspirate

INTRODUCTION

With recent advances in neonatal care, there is improved survival of extremely preterm-born babies, although significant reductions in complications of prematurity such as bronchopulmonary dysplasia (BPD) remains lacking.[1] One of its more severe complications is pulmonary hypertension associated with BPD (BPD-PH) which carries significant high morbidity and mortality.[2] The true incidence of BPD-PH in preterm babies is unknown. While the prevalence of BPD-PH is roughly one-third of all BPD cases, it is much higher in infants with severe BPD (sBPD) (17%–43%).[3–5] Diagnosis of BPD and is made around 36 weeks of gestational age (GA)[6,7] based on respiratory support requirement, while BPD-PH can present any time from 2 to 3 months of age to in early childhood in prematurely born infants. Development of BPD-PH leads to increased number of days in the neonatal intensive care, increased number of days on ventilatory support, and oxygen requirement.[8] In addition, a large number of these babies also require tracheostomy and home ventilatory support for prolonged periods.[9] Once infants are discharged home, they continue to have high mortality and morbidity with increased hospital readmissions in the first 2 years of life.[10,11] Multiple factors including prenatal and postnatal environmental stressors such as inflammation, ventilator-associated lung injury, hypoxic and hyperoxic stress, and others have been implicated in the etiology of BPD-PH.[5,12]

Severity of BPD is defined based on the therapeutic need for respiratory support at 36 weeks postmenstrual age (PMA) and its outcomes.[6,7,13] The clinical phenotype of sBPD is variable based on parenchymal pathology, airway inflammation, and pulmonary vascular disease. Wu et al. recently showed that greater than 60% of infants with sBPD had pulmonary vascular disease.[14] Due to the complex array of contributing factors, the prevention, clinical diagnosis, and effective management of BPD-PH are challenging. The development of lung alveoli is dependent on pulmonary vascular development, and any disruption of vascular growth and signaling leads to reduction in alveolar development.[15,16] Studies have also indicated that postnatal imbalance between pro- and antiangiogenic factors triggered by inflammation, and oxidative and hypoxic stressors also contribute to the development of BPD-PH.[17–19] While maternal pre-eclampsia, chorioamnionitis, and small for GA are identified as the predominant risk factors for developing BPD-PH[17,20] and could potentially predict its development, these are also factors are also key drivers of development of sBPD. This limits their potential to be used as specific markers for BPD-PH prediction, thus novel markers are needed.

Although there have been some indications of hypoxic stress injury and vascular growth factors playing a role in the development of BPD-PH, there is currently a critical gap in our knowledge of the specific molecular pathways and mechanisms involved. In the current study, we conducted a pilot unbiased approach to study multiomics profile (proteomics, microRNA [miRNAs], transcriptomics) of tracheal aspirates of infants with sBPD and BPD-PH to identify key biomarkers resulting from convergence of multiple pathophysiological processes differentiate both conditions.

METHODS

Subjects and samples

Once approved by the Penn State Health institutional review board (STUDY 00000482), infants born ≤28 weeks of gestation were screened, and informed consent obtained from parents. We included infants with diagnosis of sBPD type II[13] or grade 3 BPD based on 2019 NICHD/NRN classification.[7] Infants with congenital anomalies, chromosomal syndromes, and cyanotic cardiac defects were excluded. BPD-PH was defined based on echocardiogram features of elevated right-sided pressures including interventricular septal flattening, tricuspid regurgitation jet velocity and annular plane systolic excursion, and pulmonary artery acceleration time. Tracheal aspirate (TA) was collected from 46 infants on ventilator, by instilling 1 mL of normal saline and suctioning of endotracheal secretions. Samples were immediately stored at −80°C. Relevant clinical information was obtained by chart review. Echocardiogram reports from all infants which were obtained between 33

and 44 weeks PMA as part of routine clinical care were obtained from electronic medical records. Samples were grouped into sBPD ($n = 25$) and BPD-PH ($n = 21$) based on the echocardiogram signs of pulmonary hypertension (defined as one or more of these following signs of elevated right-sided pressures with either one or more of these findings: interventricular septal flattening, tricuspid regurgitation jet velocity, tricuspid annular plane systolic excursion, and pulmonary artery acceleration time).

MiRNA profiling

MiRNAs were extracted and analyzed from 500 μL of TAs and analyzed with a miRNA array (QIAGEN) as described previously.[21] Datasets were uploaded to GEO: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE205138. The top differentially expressed miRNAs were validated in a subset of samples ($n = 17$), with commercial miRNA assays (GeneCopoeia) and hsa-miR-16-1-3p as normalization control.[22]

Transcriptomics analysis

Library preparation and sequencing were performed from a sample subset due limited amount of original sample availability, sBPD ($n = 15$) and BPD-PH ($n = 7$), at the Penn State Health genomics core as previously described.[21] Sample counts were uploaded to GEO (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE156028). The expression of the top differentially expressed gene (IL6) was validated by qPCR (quantitative real time polymerase chain reaction) in a subset of samples (sBPD = 7, BPD-PH 8).

Proteomics analysis

TAs containing 10 μg of protein were pooled and iTRAQ labeled according to published protocols[23] at the Penn State Health proteomics core. Peptides were identified and quantified using the Paragon Algorithm in ProteinPilotTM 4.5 beta software (ABSciex).[24] Stringent local false discovery rate (FDR) estimation was calculated from the Proteomics System Performance Evaluation Pipeline algorithm.[25]

Data analysis

For miRNA arrays, statistical analyses were performed with the R software using the Bioconductor limma package. For miRNA validation analysis, differential expression was calculated using the $2^{-\Delta\Delta C_T}$ method,[26] and significant differences determined by Student's t test using GraphPad Prism. For transcriptomics, filtered reads were aligned to the human reference genome (GRCh38) using HISAT2 (version 2.1.0),[27] and the resulting deduplicated reads were summarized to each gene using HTSeq.[28] Differential gene expression analysis was conducted with the edgeR package on R,[29] after normalizing data with the TMM method.[30] Heatmaps were generated with the nonnegative matrix factorization package.[31] For proteomics, data was analyzed using the Scaffold Q+ for peptide and protein identifications, and probabilities were assigned by the Protein Prophet algorithm.[32] Channels were corrected in all samples according to the i-Tracker algorithm.[33] Normalization was performed iteratively on intensities, as described in statistical analysis of relative labeled mass spectrometry data from complex samples using analysis of variance.[34]

Pathway analysis

The ingenuity pathway analysis (IPA) software (QIAGEN) was used to identify miRNA, mRNA and protein interaction networks, predicted target genes, and molecular functions based on prediction scores.[35] The core analysis functionality was performed for direct and indirect relationships using the Ingenuity knowledge base.

RESULTS

Patient demographics

TAs from 46 preterm infants needing mechanical ventilation support were collected. Of these, 25 infants had sBPD without evidence of BPD-PH on echocardiogram and 21 infants had clinical features characterized as sBPD with evidence of BPD-PH on echocardiogram. A summary is shown in Table 1. No statistically significant differences were found for measured variables including GA at birth, birth weight, day of life at sample collection time, male sex, and racial/ethnicity group. Birth delivery by C-section was higher in the sBPD group compared with BPD-PH. GA at the time of sample collection was significantly higher in the BPD-PH group (Table 1) since they had needed longer periods of mechanical ventilation.

TABLE 1 Patient demographics at study enrollment.

Characteristic	sBPD ($n = 25$)	BPD + PH ($n = 21$)	p Value
GA at birth, weeks (mean ± SD)	25.88 ± 1.53	25.41 ± 1.82	0.345
GA at sample collection, weeks (mean ± SD)	36.15 ± 10.88	44.06 ± 14.73	0.042
Birth weight, grams (mean ± SD)	747 ± 206	647 ± 208	0.112
Male sex, % (n)	56 (14)	62 (13)	0.345
Fractional inspired oxygen during sample collection (mean ± SD)	0.37 ± 0.13	0.35 ± 0.10	0.572
Delivered via C-section, % (n)	88 (22)	43 (9)	0.002
Racial/ethnic group, % (n)			
Non-Hispanic White	52 (13)	57 (12)	0.845
Non-Hispanic Black	4 (1)	5 (1)	1.0
Hispanic	24 (6)	14 (3)	0.477
Asian	12 (3)	0 (0)	0.240
More than one race	8 (2)	24 (5)	0.220

Abbreviations: BPD, bronchopulmonary dysplasia; GA, gestational age; sBPD, severe BPD.

MiRNA analysis

The miRNA analysis was conducted in 47 samples (21 BPD-PH and 25 sBPD). A total of 1059 miRNAs were expressed in TA samples from sBPD and BPD-PH (Figure 1a). A list of miRNAs is shown in Supporting Information: Table E1, and a volcano plot is shown in Figure 1b. After accounting for FDR < 0.05, the polymerase chain reaction array analysis revealed differential expression of 12 miRNAs with |FC (fold change)| > 2 between the BPD-PH and sBPD samples (Table 2a). Of these, nine miRNAs (hsa-miR-29a, hsa-miR-542-3p, hsa-miR-624*, hsa-miR-3193, hsa-miR-3672, hsa-miR-3683, hsa-miR-501-3p, hsa-miR-101*, and hsa-miR-3128) had significantly higher expression in the BPD-PH group, compared with the sBPD group, whereas three miRNAs (hsa-miR-183*, hsa-miR-3131, and hsa-miR-1287) had significantly lower expression in BPD-PH versus sBPD. Validation qPCR experiments in a select subset of samples confirmed upregulation of hsa-miR-29a, hsa-miR-542-3p, hsa-miR-624*, hsa-miR-501-3p and hsa-miR-101*, and downregulation of hsa-miR-183* in BPD-PH compared with sBPD samples (Figure 1c).

Transcriptomic analysis

The RNA-seq analysis in 22 samples (7 BPD-PH and 15 sBPD) detected expression of 64,253 total transcripts in TAs. Of these, 31,420 transcripts showed counts >10. A heatmap of gene expression is shown in Figure 2a, and a volcano plot is shown in Figure 2b. A total of 1584 genes displayed differential expression between groups (|logFC| > 2, $p < 0.05$), including 221 with higher expression in BPD-PH versus sBPD and 1003 with lower expression in BPD-PH versus sBPD (Supporting Information: Table E2). After correction for multiple comparisons, only six genes met the more stringent criteria for FDR < 0.1 (IL6, RPL35P5, HSD3B7, RNA5SP215, OR2A1-AS1, and RNVU1-19) (Table 2b). Of these, IL6 had the highest differential expression and lower FDR, and also considering we had limited quantity of original samples, we validated only IL-6 expression using qPCR in a subset of samples (Figure 2c).

Proteomics analysis

Proteomics analysis was conducted in a subset of TA samples derived from seven BPD-PH and five sBPD infants. A total of 712 different proteins were identified using the ProteinPilot software. Of these, 64 were significantly different between groups (Mann–Whitney test $p < 0.05$) (FDR < 0.2). Eight proteins had at least twofold change in the BPD-PH group compared with the sBPD group (Table 2c).

Pathway analysis

IPA analysis of differentially expressed miRNAs in BPD-PH versus sBPD revealed significant associations with cell-to-cell signaling and interaction, cellular assembly and organization, and cellular function and maintenance. The

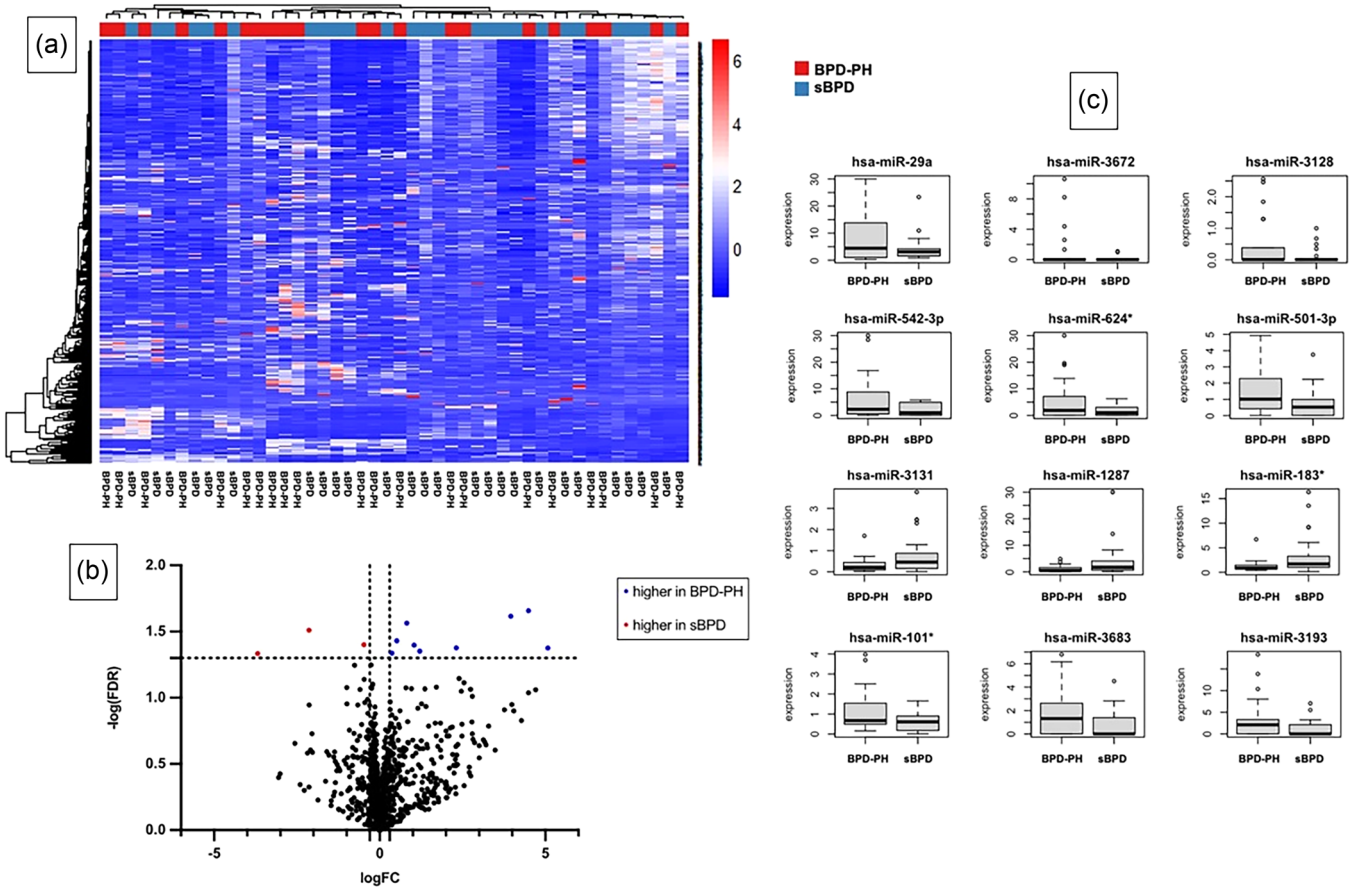

FIGURE 1 (a) MicroRNA (MiRNA) expression in tracheal aspirates. Heatmap of expression of 1048 miRNAs in tracheal aspirates from infants with severe bronchopulmonary dysplasia (sBPD) ($n = 25$) and pulmonary hypertension associated with BPD BPD-PH) ($n = 21$) (normalized by global mean), obtained by polymerase chain reaction (PCR) arrays. Heatmap generated in R using the NMF package. (b) Volcano plot. -Log of false discovery rate versus log of fold change of miRNAs between BPD-PH and sBPD groups. Vertical lines show |FC (fold change)| = 2 and horizontal line indicates false discovery rate = 0.05. (c) MiRNA validation of top differentially expressed miRNAs. Validation of miRNA expression by real time PCR. *Y* axis indicate relative expression values after normalization to miR-16, and a BPD-PH calibration sample using the $2^{-\Delta\Delta C_T}$ method.[22,26] Significant differences were determined by *t* test using the GraphPad software (*$p < 0.05$).

TABLE 2a Differentially expressed miRNAs BPD-PH versus sBPD in tracheal aspirates.

miRNA ID	Log (FC)	Average expression	Adjusted *p* value
hsa-miR-542-3p	4.487	4.098	0.022
hsa-miR-624*	3.959	3.510	0.024
hsa-miR-501-3p	0.817	1.105	0.027
hsa-miR-183*	−2.135	2.509	0.031
hsa-miR-101*	0.518	0.843	0.037
hsa-miR-3131	−0.476	0.574	0.040
hsa-miR-3683	1.037	1.290	0.040
hsa-miR-3193	2.307	2.372	0.042
hsa-miR-29a	5.077	6.423	0.042
hsa-miR-3672	1.207	0.644	0.044
hsa-miR-3128	0.369	0.282	0.046
hsa-miR-1287	−3.686	3.155	0.046

Abbreviation: FC, fold change.

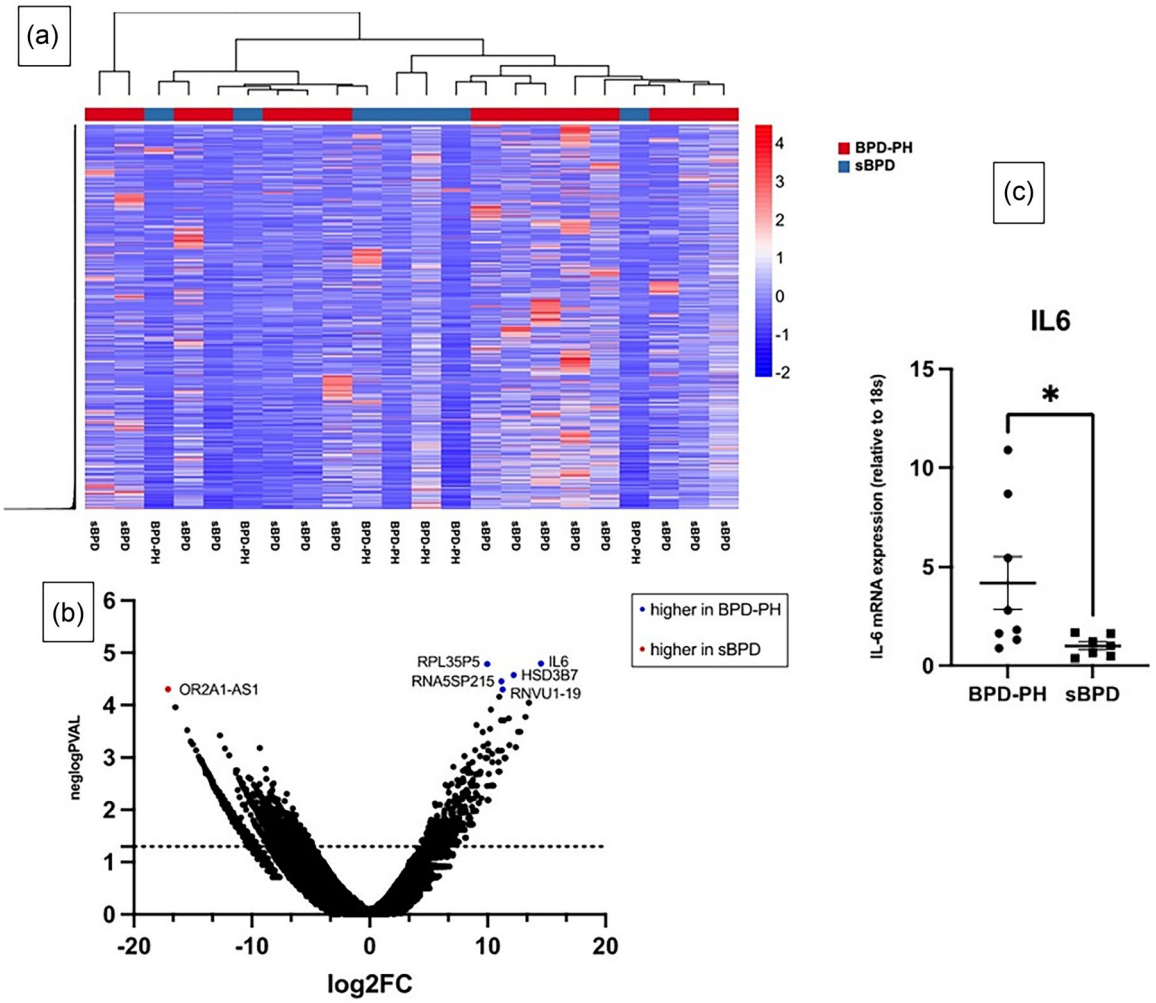

FIGURE 2 (a) Transcriptomic analysis of tracheal aspirates. Heatmap of RNA-seq counts (>10) for transcripts in tracheal aspirate samples from pulmonary hypertension associated with BPD (BPD-PH) (P, $n = 9$) and severe BPD (sBPD) (B, $n = 15$) infants. (b) Volcano plot of messenger RNA (mRNA) transcripts. -Log of false discovery rate versus log of fold change of mRNAs between BPD-PH and sBPD groups. Vertical lines show |FC (fold change)| = 2 and horizontal line indicates FDR = 0.05. (c) Interleukin-6 (IL-6) mRNA expression in tracheal aspirates. Validation of IL-6 expression using real-time polymerase chain reaction (PCR) shows increased expression in BPD-PH ($n = 8$) samples compared with sBPD ($n = 7$) ($p < 0.05$).

TABLE 2b Differentially expressed transcripts in tracheal aspirates of BPD-PH versus sBPD patients.

Gene ID	Gene name	Log (FC)	*p* Value	FDR
IL6	Interleukin 6	4.372	4.777	5.55E-02
RPL35P5	Ribosomal protein L35 pseudogene 5	3.002	5.788	5.55E-02
HSD3B7	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7	3.674	4.896	5.99E-02
RNA5SP215	RNA, 5S ribosomal pseudogene 215	3.362	5.230	5.99E-02
OR2A1-AS1	OR2A1 antisense RNA 1	−5.147	5.654	5.99E-02
RNVU1-19	RNA, variant U1 small nuclear 19	3.397	5.062	5.99E-02

Abbreviations: FC, fold change; FDR, false discovery rate.

top physiological pathways are associated with nervous system development and function, tissue development and cardiovascular system development and function. The top diseases and disorders associated were cancer, organismal injury and abnormalities, and gastrointestinal disease (Table 3a). IPA analysis of differentially expressed transcripts showed involvement of cellular development, cellular growth and proliferation, and cell death and

TABLE 2c Differentially expressed proteins in tracheal aspirates of BPD-PH versus sBPD patients.

Proteins	Log (FC)	Mann–Whitney test	Adjusted *p* value
Calcyphosin isoform a	−2.24	0.021	0.041
Alpha-1-antitrypsin precursor	−2.26	0.005	0.055
Keratin, type II cytoskeletal 5	−2.42	0.034	0.094
Pulmonary surfactant-associated protein B precursor	−3.11	0.009	0.110
Galectin-3-binding protein precursor	−4.04	0.020	0.114

Abbreviation: FC, fold change.

survival. The top physiological pathways were associated with embryonic development, hematological system development and function and hematopoiesis, and the top disease and disorders associated were cardiovascular disease, developmental disorders, and hereditary disorder (Table 3b). Similarly, IPA analysis of differentially expressed proteins identified molecular and cellular functions involving cellular compromise, protein synthesis, and cell death and survival. The top physiological pathway associated with identified proteins was tissue morphology, visual system development and function, and organ morphology. The top diseases and disorders associated were inflammatory response, cancer, and organismal injury and abnormalities (Table 3c). Finally, the integrated pathway analysis of differentially expressed miRNAs, mRNAs and proteins highlighted NFkB complex, VEGF, SERPINA1, as well as insulin, LDL, ERK1/2, and IL6 as the affected pathways in BPD-PH when compared with sBPD (Figure 3).

TABLE 3a Differentially expressed miRNA in BPD-PH versus sBPD.

	p Value range	Number of molecules
Molecular and cellular functions		
Cell-to-cell signaling and interaction	1.27E-03–5.08E-04	2
Cellular assembly and organization	2.03E-03–5.08E-04	2
Cellular function and maintenance	1.54E-02–5.08E-04	2
Cellular development	4.95E-02–8.44E-04	4
Cellular growth and proliferation	4.95E-02–8.44E-04	4
Physiological system development and function		
Nervous system development and function	8.87E-03–5.08E-04	2
Tissue development	1.54E-03–5.08E-04	3
Cardiovascular system development and function	1.27E-03–1.27E-03	1
Connective tissue development and function	4.56E-03–1.27E-03	2
Hematological system development and function	1.54E-03–1.27E-03	1
Diseases and disorders		
Cancer	4.47E-02–1.56E-04	5
Organismal injury and abnormalities	4.47E-02–1.56E-04	6
Gastrointestinal disease	4.20E-02–3.19E-04	5
Respiratory disease	3.19E-04–3.19E-04	2
Tumor morphology	2.54E-03–1.02E-03	1

DISCUSSION

Pulmonary hypertension associated with BPD is a multifactorial disease with high morbidity and mortality.[36] The mechanisms that differentiate the pathophysiology of sBPD and BPD-PH development remain unknown, which limits our ability to properly diagnose and treat infants.[37] Using an unbiased multiomic approach, our study has identified a novel biomarker panel including 12 miRNAs, 6 transcripts, and 5 proteins which distinguish infants with BPD-PH from those with sBPD who lack detectable PH on echocardiogram. Target molecules identified via IPA are NFkB, VEGF, SERPINA1, IL6, ERK1/2, and insulin to name a few.

Identifying disease endotypes of sBPD especially those with pulmonary vascular disease using biomarkers will help not only identify underlying mechanistic pathways, but also help clinically stratify high-risk infants to help improve long-term outcomes. For this approach to have translational value, it is important to identify an affordable, minimally invasive, biochemical marker to complement other factors such as Intrauterine growth restriction, maternal chorioamnionitis, and early echocardiogram findings to identify high-risk infants. Our study using TA samples in preterm infants leverages a noninvasive easily obtained biofluids.

TABLE 3b Differentially expressed mRNA in BPD-PH versus sBPD.

	p Value range	Number of molecules
Molecular and cellular functions		
Cellular development	6.29E-03–6.69E-06	3
Cellular growth and proliferation	6.29E-03–6.69E-06	3
Cell death and survival	6.04E-03–4.12E-05	3
Cell morphology	5.79E-03–6.46E-05	2
Cell-to-cell signaling and interaction	5.54E-03–2.52E-04	1
Physiological system development and function		
Embryonic development	6.29E-03–6.69E-06	3
Hematological system development and function	6.29E-03–6.69E-06	3
Hematopoiesis	6.29E-03–6.69E-06	3
Lymphoid tissue structure and development	6.29E-03–6.69E-06	3
Organ development	6.29E-03 – 6.69E-06	3
Diseases and disorders		
Cardiovascular disease	5.28E-03–1.22E-05	3
Developmental disorder	3.53E-03–1.22E-05	2
Hereditary disorder	2.52E-03–1.22E-05	2
Neurological disorder	5.79E-03–1.22E-05	2
Organismal injury and abnormalities	6.29E-03–1.22E-05	5

TABLE 3c Differentially expressed proteomics in BPD-PH versus sBPD.

	p Value range	Number of molecules
Molecular and cellular functions		
Cellular compromise	2.41E-02–5.94E-10	8
Protein synthesis	2.18E-02–2.71E-06	7
Cell death and survival	2.10E-02–1.82E-05	9
Cell morphology	1.63E-02–2.70E-05	8
Cellular movement	2.32E-03–2.70E-05	7
Physiological system development and function		
Tissue morphology	1.76E-02–6.03E-06	6
Visual system development and function	6.04E-07–6.04E-07	3
Organ morphology	2.36E-02–9.02E-06	5
Organismal development	2.36E-02–9.02E-06	7
Reproductive system development and function	2.04E-02–9.02E-06	2
Diseases and disorders		
Inflammatory response	2.32E-02–5.94E-10	10
Cancer	2.29E-02–6.03E-07	12
Organismal injury and abnormalities	2.45E-02–6.03E-07	12
Reproductive system disease	2.43E-02–6.03E-07	8
Developmental disorder	1.99E-02–7.85E-06	9

Multiomics approaches have been used to understand complex pulmonary disease such as asthma, COPD, ARDS, IPF, and PAH to develop personalized diagnostics and treatments.[38] While some studies have used arginine metabolites to endotype pulmonary arterial hypertension,[39] we chose to conduct a nontargeted approach, given the complex array of prenatal and postnatal stressors associated with BPD-PH resulting in arrested pulmonary vascular development. While antenatal and perinatal factors including intrauterine growth restriction, pre-eclampsia, maternal chorioamnionitis, and hypoxic and hyperoxic stress responses lead to abnormal pulmonary vasculogenesis and alveolarization that have been associated with sBPD, BPD-PH presents with vascular disease in its most severe form.[40,41] This suggests that BPD-PH is a more severe spectrum of sBPD phenotype.

Our analysis revealed nine miRNAs that were upregulated in BPD-PH when compared with sBPD. Of particular interest, miRNA hsa-miR-29a, which is upregulated in BPD-PH, has been previously linked with cardiovascular diseases. A publication by Chen et. al showed upregulation of miRNA 29 by 16α-Hydroxyestrone resulting in BMPR2-associated pulmonary hypertension both in human and animal models.[42] Moreover, miRNA-29a is also a well-studied biomarker for hypertrophic cardiomyopathy,[43] and thus could indicate myocardial stress as a result of elevated pulmonary pressure. Another study has noted microvesicle-secreted miR-29a/c significantly suppresses VEGF expression in gastric cancer, inhibiting vascular cell growth.[44] Contradicting, in a pre-eclampsia model of human umbilical vein endothelial cells, knockdown of miRNA 29a/c-3p inhibited VEGF2 and FGF2-induced endothelial migration.[45] Bhatt and colleagues demonstrated reduced expression of VEGF mRNA along with VEGF receptor in preterm infants with fatal lung

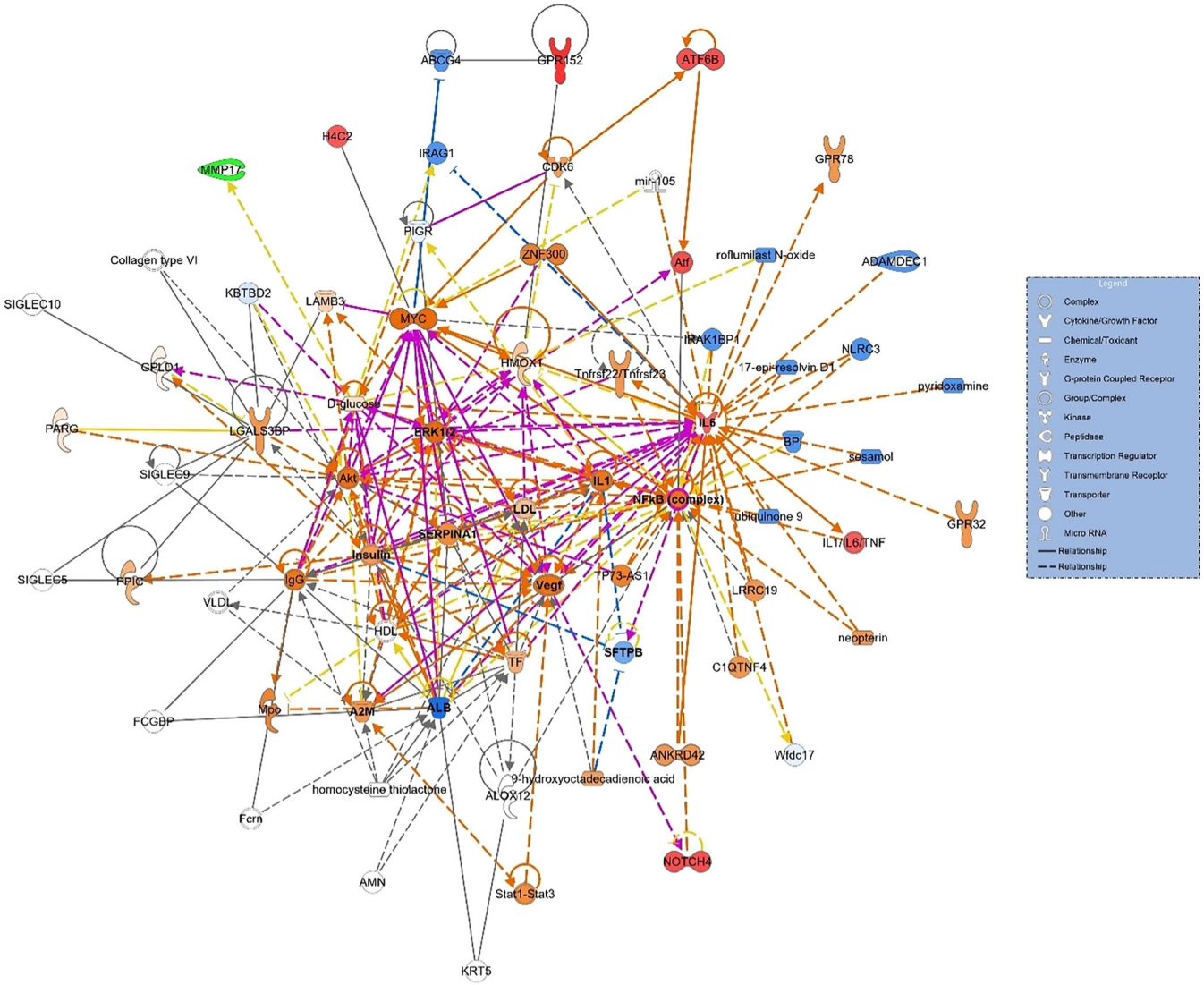

FIGURE 3 Ingenuity pathway analysis (IPA) pathways. IPA of the differentially expressed micro RNAs, proteins, and messenger RNAs in tracheal aspirates of severe bronchopulmonary dysplasia and pulmonary hypertension associated with BPD.

disease,[46] while Lassus and colleagues demonstrated lower VEGF in preterm infants with persistent severe lung disease when compared with those who recovered.[16] Whether miRNA 29a is a biomarker of VEGF expression or cardiac stress in preterm infants with BPD-PH needs further exploration. But it is a potential biomarker of interest to assess disease prediction and prognosis in future studies.

Another miRNA highly expressed in BPD-PH versus sBPD was hsa-miR-542-3p. This miRNA has been shown to target BMP7 and regulate osteogenic transition of vascular smooth muscle cells in aging and hepatic fibrosis in rats.[47,48] Additionally, of the three down-regulated miRNAs, hsa-miR-183 is typically expressed as a cluster miR-183-96-82 and packaged in exosomes expressed in high levels in cancer types including breast and prostate.[49] This miRNA functions as an oncogene by targeting transcription factor EGR1 and promote tumor migration[50] and potentially inhibits NFKB-1 expression by directly targeting its 3′-untranslated region.[51] Similarly, hsa-miR-101-5p has been studied to regulate cell proliferation through inhibition of RAP1A, which is involved in embryonic blood vessel formation.[52] This miRNA has also been shown to inhibit growth, proliferation, and migration through targeting sex-determining region Y- box 2 (SOX2).[53] Overall, our miRNA analysis revealed multiple potential key players of the mechanisms involved in BPH-PH progression.

While our transcriptomic analysis identified multiple pseudogenes, and noncoding RNAs as the top differentially expressed transcripts between BPD-PH and sBPD, it also revealed IL6 as a highly expressed gene in TAs from infants with BPD-PH. Previously, a multicenter study identified IL6 as a clinical marker to predict severe

phenotype in pediatric pulmonary arterial hypertension.[54] This cytokine has been found to modulate expression of BMPR2[55] similar to miRNA 29a which plays a role in vascular remodeling.[56] In addition, IL6 is shown to be an important clinical marker for clinical phenotype and survival in patients with pulmonary hypertension and could been a potential prognostic marker of BPD-PH severity and prognosis. Another differentially expressed gene identified was HSD3B7 which has been shown to regulate endometrial activity in women with polycystic ovarian syndrome[57] who's novel mutation is found to be associated with neonatal cholestasis.[58] In addition to these, we identified several pseudogenes which are novel and needs to be yet studied.

Finally, our proteomic analysis revealed Galectin 3 binding protein precursor gene as the most differentially expressed protein. Galectin is an important mediator of VEGF and bFGF-mediated angiogenic response which has been shown to be reduced in the presence of Galectin 3 inhibitors.[59,60] Another protein of interest was Surfactant protein B, as gene polymorphisms of SFPB have shown to be associated with BPD and newborn respiratory distress syndrome.[61,62]

The IPA analysis combining the multiomic panel compared pretranscriptional, transcriptional and post-transcriptional biomarkers helped identify key target molecules in pathobiology of BPD-PH including NFkB complex, VEGf, SERPINA1, IL6, ERK1/2, NOTCH4, A2M among many others.

Some of the strengths of our study are our non-biased approach to identify biomarkers to endotype preterm infants with sBPD versus BPD-PH. Using a multiomic approach helped us identify coding and noncoding, as well as transcriptional, and posttranscriptional markers. We used bioinformatic tools to identify key pathways and mechanisms converging and comparing the miRNAs, mRNA, and proteomic data. Given the complex pathophysiology of sBPD and BPD-PH, our study combining biological fluids with a multiomic approach identified predicted targets that have been culminated by various pathways resulting in sBPD and BPD-PH phenotypes. To ensure interreporting bias, all echocardiograms were reviewed by a single pediatric cardiologist. We excluded extremely low gestational age newborns (ELGANs) with hemodynamically significant intracardiac shunt in the study to eliminate confounding factors resulting from pulmonary over circulation or sheer stress on pulmonary vasculature.

Despite our findings, this study is not without limitations. As a pilot study, the size of the study cohort leaves opportunity for a larger follow-up study. However, while a study size of 46 preterm infants seems like a smaller cohort, it is very challenging to recruit and conduct sample analysis from ELGANs especially those who are invasively ventilated, since the clinical preference is to noninvasively ventilate which limits our access to TA samples. Echocardiograms were used as the diagnostic tool, which could be criticized as it lacks the sensitivity and specificity of the gold standard cardiac catheterization; however, we point out the limitation of reliance on invasive cardiac catheterization in extreme preterm infants which is a population less likely to undergo cardiac catheterization. There have been multiple studies validating specific echocardiogram parameters with high correlation with cardiac catheterization.[63–65] Identifying pathological biomarkers would help increase the sensitivity and specificity of noninvasive tools such as echocardiograms which measure indirect evidence of pulmonary vascular resistance. In addition, in our sample analysis of TA, we extracted total RNA which included RNA in the fluid, RNA from cell lysis during storage, and RNA from extracellular vesicles and hence we were unable to specifically identify the cellular origin of the biomarkers. Another limitation of our study is that the age of sample collection in BPD-PH group was slightly older (44 vs. 36 weeks PMA) than the infants with sBPD which could confound our results. Given the varying "n" for each "omic" profile based on the sample availability and yield, we also had to use different "cut-off" for correction for FDR which was based on the number of samples and the number of mRNAs, miRNAs, and proteins isolated from these samples.

Exosomes are membrane-bound vesicles (30–150 nm in diameter) that shuttle various molecules involved in cell-to-cell communication. TA are enriched with exosomes and microvesicles released from alveolar and endothelial cells that are isolated as early as 3–7 days of age in ELGANs that are associated with lung development and disease pathogenesis.[66,67] Endothelial cells are in close proximation alveolar epithelial cells lining the alveolar lumen with ongoing cell-to-cell signaling between two cell lineage that is crucial for lung and vascular development. While our study did not isolate the exosomes or identify the source of the exosomes before conducting multiomic analysis, our panel represents the disease-specific signature in TA specific for pulmonary vascular disease associated with BPD. In addition, while our current panel identified potential markers, we need further studies to identify their predictive and prognostic values of these markers.

In conclusion, there is a significant overlap in the pathophysiology of sBPD and BPD-PH along with their clinical signs and symptoms. Our study evaluated and identified specific multiomic markers in TA of ELGANS that aid in biochemical characterization of pulmonary vascular disease associated with BPD in these infants. We

report specific miRNAs, transcripts, and proteins that could potentially serve as target markers to evaluate predictive and prognostic value in future studies to complement clinical markers.

AUTHOR CONTRIBUTIONS

Roopa Siddaiah: Conception; research design; sample collection; data interpretation; manuscript preparation and final approval of manuscript. **Christiana Oji-Mmuo**: Research design; sample collection; data interpretation; critical revision of manuscript. **Vincent Aluquin**: Research design; sample collection; data interpretation; critical revision of manuscript. **Yuka Imamura Kawasawa**: Research design; sample collection; data interpretation; critical revision of manuscript. **Ann Donnelly**: Research design; sample collection; data interpretation; critical revision of manuscript. **Dustin Rousselle**: Research design; sample collection; data interpretation; critical revision of manuscript. **Nathalie Fuentes**: Research design; sample collection; data interpretation; critical revision of manuscript. **Eric D. Austin**: Research design; sample collection; data interpretation; critical revision of manuscript. **Patricia Silveyra**: Conception; research design; data analysis; data interpretation; sample processing; manuscript preparation.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE205138 and https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE156028.

ETHICS STATEMENT

The study is approved by the Penn State Health Institutional Review Board 'STUDY 00000482'.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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