NOVEL BIOMARKERS OF BRONCHOPULMONARY DYSPLASIA AND BRONCHOPULMONARY DYSPLASIA-ASSOCIATED PULMONARY HYPERTENSION

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Abstract

Objective—To quantify and compare levels of potential biomarkers in neonates with i) Bronchopulmonary dysplasia (BPD); ii) BPD-associated pulmonary hypertension (BPD-PH); iii) PH without BPD; and iv) neonates without lung disease at ~36 weeks postmenstrual age.

Study Design—Multiple potential biomarkers were measured in plasma samples of 90 patients using a multi-spot enzyme-linked immunosorbent assay. Statistical tests done included one-way ANOVA to compare levels of biomarkers between different groups.

Results—Higher levels of ICAM-1 were present in infants with BPD and correlated with its severity. Infants with BPD have significantly higher levels of ANG-2 and lower levels of ANG-1. Infants with PH have higher levels of: IL-6, IL-8, IL-10, and TNF-α. Infants with BPD-PH, have significantly lower levels of MCP-1 and higher levels of IL-1β than in infants with PH without BPD.
Conclusion—ICAM-1 may be used as a specific biomarker for diagnosis of BPD and its severity.

Introduction

Bronchopulmonary dysplasia (BPD) and BPD-associated pulmonary hypertension (BPD-PH) are chronic inflammatory cardiopulmonary diseases with devastating short and long-term consequences. BPD is the most common chronic lung disease in premature infants and its incidence differs between various centers based on the perinatal practices and the definition used for diagnosis. We used the NIH consensus definition for identifying infants with BPD as this definition has been validated in other studies and it allowed us to identify infants with established BPD at 36 weeks post menstrual age (PMA). We evaluated the level of potential biomarkers at 36 weeks PMA in order to assess their utility as an additional aid for BPD diagnosis.

A biomarker is a guide to the pathological condition and should have the capacity to be detected in the diseased state or prior to the development of disease. Various studies have evaluated biomarkers associated with lung injury as a potential source for predicting the infants at risk for BPD. Biomarkers have been studied for BPD in cord and peripheral blood, tracheal aspirates and urine. However, most studies have been done in the first few weeks of the infant’s life. No studies have evaluated their utility at the time of assessment for BPD, for the purpose of diagnosis and assessment of disease severity.

Infants with BPD are also predisposed to abnormal growth of pulmonary vasculature with dysregulated pulmonary vascular density and increased pulmonary vascular resistance. Chronic hypoxia in infants with BPD also leads to vascular remodeling with intimal hyperplasia in this dysregulated pulmonary vasculature, which contributes to pulmonary hypertension (PH) in infants with BPD. There is no reliable biomarker that can help diagnose PH in these infants and monitor response to therapy in infants with BPD-PH. Identification of a biomarker that can be used in the setting of BPD to identify the infants most at risk for developing PH will greatly facilitate the diagnosis and management of these infants.

Based on the pathogenesis of BPD and data from previous animal and human studies, we identified various proteins that had the potential for acting as a biomarker for BPD and its complications such as PH. Ion channels play a major role in PH and one such channel implicated in adult PH is chloride intracellular channel (CLIC) protein. Our objective in this study was to quantify and compare the levels of these potential inflammatory modulators and ion channels as biomarker protein in neonates with established BPD, BPD-PH, PH without BPD and in term/preterm neonates without any known lung pathology. To determine factors affecting these potential biomarkers, we also compared the antenatal and perinatal factors associated with the biomarker levels.
Patients and Methods

Study Sites and Patient Enrollment Criteria:

This study was performed at 3 neonatal intensive care units (NICUs), a level III and a Level IV academic NICU in Philadelphia, PA and a level IV academic NICU in Columbus, OH. The institutional review boards at Drexel University College of Medicine and Nationwide Children’s Hospital approved the study with waiver of consent. This study was designed as a proof of concept. The infants included in the discovery cohort (Philadelphia cohort: BPD-SC) of the study were divided into 5 groups based on their pathology. The 5 groups included, preterm infants with no BPD (PC=9), term infants with no lung pathology/BPD (TC=17), preterm infants with BPD (BPD=25), term infants with PH (PH=7), infants with BPD who developed PH (BPD-PH=3). Supplemental Table 1 illustrates the inclusion criteria for each group along with the number of subjects. The sample size was determined by the number of infants that met the eligibility criteria during the study period. Infants with congenital anomalies and genetic disorders affecting the cardio-pulmonary system were excluded. To validate the results of the biomarker profile obtained at 36 weeks PMA in the discovery cohort, plasma samples were obtained from the validation cohort (Nationwide cohort: BPD-NW). Specific biomarkers were analyzed in plasma samples of infants with severe BPD from the validation cohort from Nationwide Children’s Hospital, Columbus, OH (BPD-NW) and compared with preterm controls and BPD group from the discovery cohort from St. Christopher’s Hospital, Philadelphia, PA (BPD-SC). Infants with moderate and severe BPD were included.

Blood Collection:

Scavenged blood from discarded samples was obtained from neonates meeting the eligibility criteria.

ELISA assay:

Multiple cytokines and proteins were measured using a personalized Meso Scale Discovery (MSD) MULTI-SPOT assay test (Meso Scale Diagnostics, Maryland). Angiopoietin (ANG) 1 and 2 and CLICs 1 and 4 proteins were measured separately using specific ELISA kits (R & D System, Minneapolis, MN and Cloud-Clone Corp., Katy, TX respectively). Due to our limited knowledge about these biomarkers in BPD and PH, a power analysis was not done. To verify the results from the discovery cohort, specific cytokines and proteins were measured in the validation cohort using a multi-spot assay and compared with the discovery cohort of preterm controls and BPD group (moderate and severe).

Statistics:

GraphPad Prizm 7v.0 (San Diego, CA) was used for statistical analysis. Robust regression and Outlier removal (ROUT) method was used to identify and remove the outliers from the data set. ROUT Coefficient (Q) value of 0.1 was used. This allows for the removal of definite outliers from the data set. Fischer exact test was used to compare baseline maternal and neonatal characteristics. One-way ANOVA was used to compare the means of level of biomarkers between different groups. Tukey’s test was used in conjunction with ANOVA to
correct for multiple comparisons. For 2 subjects serial biomarkers were measured. These biomarker levels were compared between the time points of disease progression from BPD to BPD-PH. The difference in means of these biomarkers with disease progression was compared using the two-way ANOVA test.

Results

A total of 101 patients were recruited in the study and biomarkers from 90 patients were analyzed in the study. The discovery cohort included 50 patients. The validation cohort of the study included an additional 40 infants with BPD that were analyzed to corroborate the initial results. The study was designed to compare the PC with the BPD group and TC with the PH population. The results from the discovery cohort are discussed below-

Patient characteristics

The total number of patients enrolled in each group are: TC=17, PC=9, BPD=25, PH=7, BPD+PH=3. Of note, the infants that developed BPD had a lower gestational age (GA), lower birth weight, lower initial APGAR scores and included less small for GA (SGA) infants as compared to preterm controls. However, the PMA at which their samples were collected did not differ. The patient demographics were similar in infants with PH and TC groups, except the initial APGAR score was significantly lower in the PH group when compared to TC. Overall, infants with BPD-PH had similar demographics to infants with BPD alone. However, there were two notable exceptions. First, not surprisingly, samples collected from BPD-PH group were at an older PMA, due to PH being a later complication of BPD. Second, all infants with BPD-PH were SGA. Table 1 summarizes the comparison of patient characteristics between various groups. None of the infants enrolled in the study had a culture positive sepsis within the last 2 weeks from when the blood sample was taken. The maternal characteristics did not differ significantly between different groups. Supplemental Table 2 summarizes the comparison between selected maternal characteristics of the discovery cohort.

Biomarkers-

All biomarkers were not measured in all infants enrolled in each group due to insufficient sample size in some patient. The ‘n’ in each group for various biomarkers are listed in Table 2. Table 2 summarizes the difference in the levels of various biomarkers between different groups. The unique markers identified within various groups are discussed below.

1. **BPD group**: Higher levels of intercellular adhesion molecule (ICAM)-1 were present in infants with BPD, when compared to PC and TC. The level of ICAM-1 was also significantly elevated in infants with a diagnosis of severe BPD versus those with non-severe BPD pathology (p =0.0047). Figure 1A represents the levels of ICAM-1 with various pathologies and its correlation with severity of BPD, where we have compared levels of ICAM-1 in mild, moderate and severe BPD in the discovery cohort. As shown in Figure 1B, infants with BPD when compared to infants with BPD-PH and TC had significantly higher levels of ANG 2 and lower levels of ANG 1.
2. **BPD-PH group:** In infants with BPD-PH, levels of monocyte chemoattractant protein (MCP)-1 were significantly decreased and levels of interleukin (IL)-1β were significantly increased compared to TC and infants with PH. The levels of ICAM-1 were significantly decreased in infants who developed BPD-PH when compared to infants with BPD alone. In infants with BPD-PH, IL-6 rose significantly over time. There was also a trend of decrease in the MCP-1 and ICAM-1 levels; however, it was not significant. Figure 2 represents the levels of MCP-1 and IL-1β. Supplemental Figure 1 shows the trend in the levels of ICAM-1, MCP-1 and IL-6 in the 2 infants where serial biomarker levels were obtained between the progression from BPD to BPD-PH.

3. **PH group:** Specific cytokines [IL-6, IL-8, IL-10 and tumor necrosis factor alpha (TNF-α)] were significantly increased in infants with PH, compared to TC. Figure 3 shows the levels of these cytokines in different groups.

### Ion Channel Proteins

As shown in Supplemental Figure 2, there was no difference in the levels of CLIC-1 and CLIC-4 proteins in infants with BPD and BPD-PH when compared to controls. Although CLIC-4 has a relevant role in adult PH it does not seem to be relevant in neonates.

### Results from the validation cohort.

Table 3 summarizes the comparison of specific patient characteristics between various groups. Since neonates in the validation cohort were only moderate or severe BPD (BPD-NW), we compared them with the subgroup of BPD patient with moderate and severe BPD from the discovery cohort (BPD-SC). BPD-NW and BPD-SC groups were similar with respect to birth weight, GA and gender. All samples within the 3 groups were collected at similar PMA. Supplemental Table 3 summarizes the difference in the levels of various biomarkers between BPD-NW, BPD-SC, and PC groups. Levels of ICAM-1 were significantly elevated in infants in BPD-NW group and were similar to the levels of ICAM-1 in BPD-SC group. Figure 4A shows the levels of ICAM-1 in both BPD groups and the control group. Interestingly, the levels of other cytokines were different between the BPD-NW and BPD-SC group at 36 weeks PMA, as shown in Figure 4. BPD-NW had higher levels of IL-1β, IL-6, TNF-α and lower levels of IL-8 as compared to BPD-SC group.

### Discussion

Evaluation of biomarkers in infants with established BPD in our discovery cohort revealed high levels of ICAM-1 in the plasma samples obtained at 36 weeks PMA when compared to preterm infants without BPD at 36 weeks PMA. The level of ICAM-1 also correlated with the severity of BPD in these infants. This result was verified in the biomarker analysis done in our validation cohort. Interestingly, higher levels of IL-1β, IL-6, TNF-α and lower levels of IL-8 were seen in the BPD population of our validation cohort and were not elevated in our discovery cohort. Infants with BPD had significantly higher levels of ANG 2 and lower levels of ANG 1 when compared to infants with BPD-PH and TC. In infants with BPD that developed PH, levels of MCP-1 were significantly decreased and levels of IL-1β were...
significantly increased compared to TC and infants with PH without BPD. In infants with PH IL-6, IL-8, IL-10, and TNF-α were significantly increased when compared to TC.

As opposed to previous studies, our study is unique as we measured the levels of potential biomarkers of BPD at 36 weeks PMA. Although the initial lung injury in these infants occurs early, there is an ongoing component of repair and development that contributes to the morphology of the lungs in BPD and its complications such as PH. This is also important since many babies with BPD get transferred to referral centers at a later time for management of various comorbidities associated with prematurity and evaluation of biomarkers at this time may be pivotal to provide a helpful insight into their disease severity.

Adhesion molecules are proteins on the surface of cells which mediate transmigration of inflammatory cells from peripheral blood to their site of action and play an important role in active T-cell mediated immune response\textsuperscript{12}. As part of our vascular injury panel, we measured ICAM-1 which promotes the attachment of inflammatory cells to the endothelium, which is critical for vascular development and is a key regulator of lung maturation, airway branching, and angiogenesis\textsuperscript{13}. ICAM-1 was higher at 3 to 7 days of life in plasma samples of patients that develop BPD than in patients who do not develop BPD, suggesting that ICAM-1 expression is enhanced in lungs of patients susceptible to BPD\textsuperscript{14}. ICAM-1 is upregulated in animal models of hyperoxia induced lung injury\textsuperscript{15}. In the present study, ICAM-1 levels were significantly higher in infants with BPD at 36 week PMA when compared to both preterm and term controls and its levels were associated with the severity of BPD. This suggests that ICAM-1 contributes to the BPD pathogenesis after the initial inflammatory response in BPD and may play a role in the dysregulated lung and vascular development. We verified this result in our validation cohort, where ICAM-1 levels were significantly higher in patients with severe BPD from Nationwide Children’s Hospital. Thus, ICAM-1 levels are elevated at the time of diagnosis of BPD despite differences in patient population and practices at different institutions. If confirmed by larger studies, ICAM-1 can thus be potentially used as a novel biomarker to aid in diagnoses of BPD and may also be helpful in assessing the severity of BPD.

Infants in the BPD-NW group had higher levels of IL-1β, IL-6, TNF-α and lower levels of IL-8 when compared to the preterm controls. In a study of 1067 infants by the National Institute of Child Health and Development (NICHD) neonatal research network, 25 cytokines were measured in the blood at 3,7,14 and 21 days of life. Higher peaks of IL-1β, IL-6, IL-8, and IL-10 were associated with a combined outcome of BPD/death\textsuperscript{16}. Interestingly, this pattern of cytokines was not present in our BPD-SC group. This variability may be explained by the differences in patient population and clinical practices at different institutions. Although specific data regarding the race was not collected during the study, the patient population included in BPD-SC group is predominantly African-American (\textgreater90\%), whereas that in BPD-NW group is predominantly Caucasian (63\%) and may contribute to the differential cytokine profile seen in these patients.

ANG −1 and −2 play an important role in stabilization and destabilization of endothelium, respectively\textsuperscript{17}. ANG-2 destabilizes the endothelium, making it more responsive to new vessel sprouting and in the process increases vascular leakage\textsuperscript{18}. Increased levels of ANG-2
was present in plasma and alveolar edema fluid in adults with acute lung injury (ALI) and pulmonary edema. Increased tracheal ANG-2 was also found in neonates that developed BPD. On the other hand, ANG-1 induces tightening of endothelial intercellular junctions, helping with stabilization and maturation of newly formed vessels. Both angiopoietins synergistically work in the process of angiogenesis. Lower levels of ANG-1 in plasma cord blood are associated with development of BPD in preterm infants. BPD patients in our discovery cohort had significantly higher levels of ANG-2 and lower levels of ANG-1 when compared to infants with BPD-PH and TC, which is consistent with the known associations of these proteins.

Biomarker analysis in infants with established BPD-PH revealed lower levels of MCP-1 and higher levels of IL-1β when compared to TC. MCP-1 (also known as CCL-2) is a chemokine that helps mediate the influx of macrophages and lymphocytes and is associated with a subacute phase of airway inflammation. In infants where serial levels were obtained, the level of MCP-1 decreased from the time of diagnosis of BPD to BPD-PH. IL-1β is a proinflammatory cytokine that is released by alveolar macrophages and can amplify the inflammatory cascade by recruiting inflammatory cells, inducing production of other cytokines and adhesion molecules, as well as stimulating fibroblast activity, thereby playing a role in fibrosis. In our study, the levels of IL-1β were higher in infants with BPD-PH despite the fact that most of the initial inflammatory process has already occurred. Potentially, levels of IL-1β may remain elevated because of fibroblast stimulation and fibrosis, an ongoing process in the development of PH in this population. In infants with BPD-PH, levels of IL-6 increased significantly over time.

In adult literature, IL-6 promotes the development and progression of pulmonary vascular remodeling and pulmonary artery hypertension (PAH) through antiapoptotic mechanisms. IL-6 plays a role in a posttranscriptional mechanism of downregulation of bone morphogenetic protein receptor type (BMPR) 2 gene, in 70% cases of familial PAH. High levels of IL-6 also activate the proliferation of pulmonary artery smooth muscle cells and promotes the conversion of pulmonary endothelial cells into pulmonary smooth muscle cells, thereby contributing to remodeling of pulmonary vasculature. Our data shows that in infants with BPD who develop PH, IL-6 rises significantly over time. Its rise may be associated with its role in the remodeling of the pulmonary vasculature. IL-6 has both pro- and anti-inflammatory properties and hence may also serve a compensatory role in protecting the lung against the harmful effects of the inflammatory process. The 2 infants with BPD from the discovery cohort who developed BPD-PH, had high levels of ICAM-1 at 36 weeks PMA and it decreased over the next 4–6 weeks. ICAM-1 levels are in general lower in the group with BPD-PH. We speculate that by the time PH has developed in these infants most of the inflammatory response and dysregulated vasculature development has occurred and thus its levels were lower.

Infants with PH in our study demonstrated higher levels of IL-6, IL-8, IL-10 and TNF-α. IL-8 helps in the recruitment of neutrophils and monocytes in the lungs and acts as a growth factor for endothelial cells as it has proangiogenic and antiapoptotic properties. High serum levels of IL-8 have been seen in adult patients with PH as described as a predictor for survival. IL-10 is released by T cells and inhibits the release of pro-
inflammatory cytokines from monocytes and macrophages, enhances the release of other anti-inflammatory mediators and plays a role in the limitation of adverse inflammatory responses\textsuperscript{30, 31}. In adults with PAH elevated levels of IL-10 were believed to serve as counter regulating mechanisms against lung inflammation\textsuperscript{28}. TNF-\(\alpha\) is another potent macrophage activator and its overproduction leads to capillary leak and parenchymal lung damage. A high serum level of TNF-\(\alpha\) is present in adults with PH\textsuperscript{32}. High levels of these cytokines in the PH group may be explained based on their known function.

Our study has several limitations. First, our sample size was relatively small, especially in the PH and BPD-PH groups. This may lead to some of the findings in the study to be anecdotal; however, this was designed as an exploratory study. Secondly, due to the heterogeneity of the BPD population and the presence of varied clinical phenotypes, the results show some variability. With a larger sample size, the results could be better stratified based on the severity of BPD. Lastly, the infants in our BPD-PH group are much older than the infants in the control group and the levels of the biomarkers may be affected by the maturity of these infants.

However, the strengths of the study include a prospective design and a systematic prospective collection of samples. Secondly, we evaluated some novel biomarkers that have not been previously assessed at the time of assessment of BPD. Thirdly, a single machine assayed almost all the cytokines at the same time, thereby reducing the chance of variability with technique and assay preparation. Lastly, the identification of ICAM-1 as a biomarker for severe BPD was confirmed in the analysis from the validation cohort.

\section*{Conclusion}
Specific patterns of biomarkers can be discerned in babies with BPD and BPD-PH.

ICAM-1 is a novel and consistent biomarker for BPD, which can be an additional aid in the diagnosis of BPD and its severity. Higher ANG-2 and lower ANG-1 levels are seen in infants with BPD and may contribute to the dysregulated vasculature seen in BPD. Higher levels of inflammatory cytokines- IL-6, IL-8, IL-10, and TNF-alpha are present in infants with PH and may be associated with the development of PH. In infants with BPD-PH, higher levels of IL-1\(\beta\) may be associated with fibroblast stimulation and development of PH.

\section*{Supplementary Material}
Refer to Web version on PubMed Central for supplementary material.

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\section*{References}
1. Bhandari A, Bhandari V. Biomarkers in bronchopulmonary dysplasia. Paediatric respiratory reviews 2013, 14(3): 173–179. [PubMed: 23523392]
2. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. American journal of respiratory and critical care medicine 2001, 163(7): 1723–1729. [PubMed: 11401896]

3. Lal CV, Ambalavanan N. Biomarkers, Early Diagnosis, and Clinical Predictors of BPD. Clinics in perinatology 2015, 42(4): 739–754. [PubMed: 26593076]

4. Piersigilli F, Lam TT, Vernocchi P, Quagliariello A, Putignani L, Aghai ZH, et al. Identification of new biomarkers of bronchopulmonary dysplasia using metabolomics. Metabolomics 2019, 15(2): 20. [PubMed: 3030433]

5. Piersigilli F, Bhandari V. Biomarkers in neonatology: the new “omics” of bronchopulmonary dysplasia. The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet 2016, 29(11): 1758–1764.

6. Bose CL, Dammann CE, Laughon MM. Bronchopulmonary dysplasia and inflammatory biomarkers in the premature neonate. Arch Dis Child Fetal Neonatal Ed 2008, 93(6): F455–461. [PubMed: 18676410]

7. O’Connor MG, Suthar D, Vera K, Slaughter JC, Maitre NL, Steele S, et al. Pulmonary hypertension in the premature infant population: Analysis of echocardiographic findings and biomarkers. Pediatric pulmonology 2017.

8. Littler DR, Harrop SJ, Goodchild SC, Phang JM, Mynott AV, Jiang L, et al. The enigma of the CLIC proteins: Ion channels, redox proteins, enzymes, scaffolding proteins? FEBS letters 2010, 584(10): 2093–2101. [PubMed: 20085760]

9. Singh H Two decades with dimorphic Chloride Intracellular Channels (CLICs). FEBS letters 2010, 584(10): 2112–2121. [PubMed: 20226783]

10. Wojciak-Stothard B, Abdul-Salam VB, Lao KH, Tsang H, Irwin DC, Lisk C, et al. Aberrant chloride intracellular channel 4 expression contributes to endothelial dysfunction in pulmonary arterial hypertension. Circulation 2014, 129(17): 1770–1780. [PubMed: 24503951]

11. Gururaja Rao S, Ponnalagu D, Patel NJ, Singh H. Three Decades of Chloride Intracellular Channel Proteins: From Organelle to Organ Physiology. Current protocols in pharmacology 2018, 80(1): 11.21.11–11.21.17. [PubMed: 30040212]

12. Ren G, Roberts AI, Shi Y. Adhesion molecules: key players in Mesenchymal stem cell-mediated immunosuppression. Cell adhesion & migration 2011, 5(1): 20–22. [PubMed: 20935502]

13. Glaser K, Speer CP. Pre and Postnatal Inflammation in the Pathogenesis of Bronchopulmonary Dysplasia In: Bhandari V (ed). Bronchopulmonary Dysplasia. Springer International Publishing: Cham, 2016, pp 55–77.

14. Ramsay PL, O’Brian Smith E, Hegemier S, Welty SE. Early clinical markers for the development of bronchopulmonary dysplasia: soluble E-Selectin and ICAM-1. Pediatrics 1998, 102(4 Pt 1): 927–932. [PubMed: 9755267]

15. Welty SE, Rivera JL, Elliston JF, Smith CV, Zeb T, Ballantyne CM, et al. Increases in lung tissue expression of intercellular adhesion molecule-1 are associated with hyperoxic lung injury and inflammation in mice. American journal of respiratory cell and molecular biology 1993, 9(4): 393–400. [PubMed: 8104435]

16. Ambalavanan N, Carlo WA, D’Angio CT, McDonald SA, Das A, Schendel D, et al. Cytokines associated with bronchopulmonary dysplasia or death in extremely low birth weight infants. Pediatrics 2009, 123(4): 1132–1141. [PubMed: 19336372]

17. Distler JH, Hirth A, Kurowska-Stolarska M, Gay RE, Gay S, Distler O. Angiogenic and angiostatic factors in the molecular control of angiogenesis. The quarterly journal of nuclear medicine : official publication of the Italian Association of Nuclear Medicine (AIMN) [and] the International Association of Radiopharmacology (IAR) 2003, 47(3): 149–161.

18. Roviezzo F, Tsigkos S, Kotanidou A, Bucci M, Brancalone V, Cirino G, et al. Angiopoietin-2 Causes Inflammation in Vivo by Promoting Vascular Leakage. Journal of Pharmacology and Experimental Therapeutics 2005, 314(2): 738. [PubMed: 15870388]

19. Bhandari V, Choo-Wing R, Lee CG, Zhu Z, Nedelrow JH, Chupp GL, et al. Hyperoxia causes angiopoietin-2-mediated acute lung injury and necrotic cell death. Nature medicine 2006, 12(11): 1286–1293.
20. Thomas W, Seidenspinner S, Kramer BW, Wirbelauer J, Kawczyńska-Leda N, Szymankiewicz M, et al. Airway angiopoietin-2 in ventilated very preterm infants: Association with prenatal factors and neonatal outcome. Pediatric pulmonology 2011, 46(8): 777–784. [PubMed: 21337734]

21. Mohamed WA, Niyazy WH, Mahfouz AA. Angiopoietin-1 and endostatin levels in cord plasma predict the development of bronchopulmonary dysplasia in preterm infants. Journal of tropical pediatrics 2011, 57(5): 385–388. [PubMed: 21131270]

22. Baier RJ, Luggins J, Kruger TE. Monocyte chemoattractant protein-1 and interleukin-8 are increased in bronchopulmonary dysplasia: relation to isolation of Ureaplasma urealyticum. Journal of investigative medicine : the official publication of the American Federation for Clinical Research 2001, 49(4): 362–369. [PubMed: 11478413]

23. Baier RJ, Majid A, Parupia H, Luggins J, Kruger TE. CC chemokine concentrations increase in respiratory distress syndrome and correlate with development of bronchopulmonary dysplasia. Pediatric pulmonology 2004, 37(2): 137–148. [PubMed: 14730659]

24. Jackson W, Laughon MM. Biomarkers of Bronchopulmonary Dysplasia In: Bhandari V (ed). Bronchopulmonary Dysplasia. Springer International Publishing: Cham, 2016, pp 129–148.

25. Kotecha S, Wilson L, Wangoo A, Silverman M, Shaw RJ. Increase in Interleukin (IL)-1β and IL-6 in Bronchoalveolar Lavage Fluid Obtained from Infants with Chronic Lung Disease of Prematurity. Pediatric research 1996, 40: 250. [PubMed: 8827773]

26. Kolb M, Margetts PJ, Anthony DC, Pitosi F, Gauldie J. Transient expression of IL-1β induces acute lung injury and chronic repair leading to pulmonary fibrosis. The Journal of clinical investigation 2001, 107(12): 1529–1536. [PubMed: 11413160]

27. Steiner MK, Syrkina OL, Kolliputi N, Mark EJ, Hales CA, Waxman AB. Interleukin-6 overexpression induces pulmonary hypertension. Circ Res 2009, 104(2): 236–244, 228p following 244. [PubMed: 19074475]

28. Groth A, Vrugt B, Brock M, Speich R, Ulrich S, Huber LC. Inflammatory cytokines in pulmonary hypertension. Respiratory Research 2014, 15(1): 47–47. [PubMed: 24739042]

29. Li A, Varney ML, Valasek J, Godfrey M, Dave BJ, Singh RK. Autocrine role of interleukin-8 in induction of endothelial cell proliferation, survival, migration and MMP-2 production and angiogenesis. Angiogenesis 2005, 8(1): 63–71. [PubMed: 16132619]

30. Sabat R, Grutz G, Warszawska K, Kirsch S, Witte E, Wolk K, et al. Biology of interleukin-10. Cytokine & growth factor reviews 2010, 21(5): 331–344. [PubMed: 21115385]

31. Jones CA, Cayabyab RG, Kwong KY, Stotts C, Wong B, Hamdan H, et al. Undetectable interleukin (IL)-10 and persistent IL-8 expression early in hyaline membrane disease: a possible developmental basis for the predisposition to chronic lung inflammation in preterm newborns. Pediatric research 1996, 39(6): 966–975. [PubMed: 8725256]

32. Soon E, Holmes AM, Treacy CM, Doughty NJ, Southgate L, Machado RD, et al. Elevated levels of inflammatory cytokines predict survival in idiopathic and familial pulmonary arterial hypertension. Circulation 2010, 122(9): 920–927. [PubMed: 20713898]
Figure 1-
Biomarkers in PC, TC, BPD, PHTN and BPD-PH (Discovery cohort): A) Elevated levels of ICAM-1 as compared to PC, TC and BPD-PH. ICAM levels also correlate with severity of BPD (p=0.0047). B) Infants with BPD have higher level of Ang2 and lower level of Ang1.
Figure 2-
Biomarkers in PC, TC, BPD, PHTN and BPD-PH (Discovery cohort): A) Higher levels of IL-1β and lower level of MCP-1 were seen in BPD-PH as compared to PHTN and, TC.
Figure 3-
Biomarkers in PC, TC, BPD, PHTN and BPD-PH (Discovery cohort): Specific cytokines (IL6, IL8, IL10 and TNFα) were significantly increased in infants with PH, compared to TC.
Figure 4-
Biomarkers in BPD (validation cohort vs. discovery cohort): A) Elevated levels of ICAM-1 in both BPD groups as compared to PC. B, C & D) Higher levels of IL-1β, IL-6 and TNF-α in BPD-NW as compared to BPD-SC and PC. E) Lower levels of IL-8 in BPD-NW as compared to BPD-SC.
Table 1:
Comparison of selected study characteristics of discovery cohort, *Mean ± SD

| Variable                        | TC (n=17) | PC (n=9)  | BPD (n=25) | PH (n=7)  | BPD+PH (n=3) | PC vs. BPD | TC vs. BPD | TC vs. PH | TC vs. BPD+PH | PC vs. BPD+PH | PH vs. BPD+PH | BPD vs. BPD-PH |
|---------------------------------|-----------|-----------|------------|-----------|--------------|------------|------------|-----------|--------------|---------------|---------------|----------------|
| Birth weight* (g)               | 3271±516  | 1175±191  | 752±245    | 2966±566  | 584±72       | 0.03       | <0.01      | 0.37      | <0.01        | 0.12          | <0.01        | 0.98           |
| Gestational age* (wk)           | 38.7±1.6  | 29.7±1.1  | 25.4±2.2   | 37.6±2.2  | 25.7±1.4     | <0.01      | <0.01      | 0.7       | <0.01        | 0.01          | <0.01        | 1.0            |
| Post Menstrual Age* (wk)        | 39.2±1.5  | 36.6±1.4  | 36.9±2.4   | 38.3±2.7  | 50.8±5       | 0.09       | 0.02       | 0.96      | <0.01        | <0.01         | <0.01        | <0.01          |
| Gender-Female (n, %)            | 8 (47)    | 8 (88)    | 12 (48)    | 1 (14)    | 2 (66)       | 0.05       | 1.0        | 0.19      | 1            | 0.45          | 0.18          | 1.0            |
| Small for Gestational Age (n, %)| 0 (0)     | 6 (66)    | 6 (24)     | 1 (14)    | 3 (100)      | 0.04       | 0.06       | 0.29      | <0.01        | 0.51          | 0.03          | 0.02           |
| APGAR-1 min (median)            | 8         | 7         | 3          | 2         | 1            | <0.01      | <0.01      | <0.01     | <0.01        | 0.02          | 0.89          | 0.83           |
| APGAR-5 min (median)            | 9         | 7         | 7          | 7         | 5            | >0.99      | <0.01      | 0.01      | <0.01        | 0.46          | 0.48          | 0.39           |
| Antenatal Steroid (n, %)        | 0 (0)     | 3(33)     | 14(56)     | 2(28)     | 3(100)       | 0.43       | <0.01      | 0.39      | <0.01        | 0.04          | 0.16          | 0.25           |
| Surfactant (n, %)               | 0 (0)     | 1(11)     | 20(80)     | 2(28)     | 3(100)       | <0.01      | <0.01      | 0.07      | <0.01        | 0.01          | 0.16          | 0.25           |

TC-term control; PC- preterm control; BPD- bronchopulmonary dysplasia; PH- pulmonary hypertension; BPD+PH- BPD associated pulmonary hypertension;
Table 2A –
Mean (±S.D.) levels of biomarkers in different groups in discovery cohort; *levels in ng/ml

| Biomarker | TC | PC | BPD | PH | BPD + PH | PC vs. BPD | TC vs. BPD | PC vs. BPD + PH | PH vs. BPD + PH |
|-----------|----|----|-----|----|----------|------------|------------|----------------|-----------------|
| pg/ml     |    |    |     |    |          |            |            |                |                 |
| IL-10     | 0.01±0.01 | 1.32±0.5 | 1.36±0.6 | 3.49±1.6 | 0.84±0.7 | 1.0 | <0.01 | 0.67 | 0.97 | <0.01 | 0.99 |
| IL-13     | <0.01 | 0.25±0.4 | 0.17±0.2 | 0.19±0.2 | 0.22±0.2 | 0.98 | 0.60 | 0.70 | 0.77 | >0.99 | >0.99 | 0.99 |
| IL-1β     | 0.03±0.03 | 0.46±0.3 | 1.38±1.3 | 0.52±0.2 | 3.38±3.9 | 0.58 | 0.12 | 0.98 | <0.01 | 0.02 | 0.02 | 0.12 |
| IL-6      | 0 | 1.19±1.5 | 2.07±1.8 | 20.21±30.2 | 13.35±7 | 1.0 | 1.00 | 0.02 | 0.53 | 0.66 | 0.96 | 0.68 |
| IL-8      | 0.02±0.01 | 41.6±17 | 95.3±72 | 103±57 | 82.4±26 | 0.25 | <0.01 | 0.01 | 0.21 | 0.92 | 1.00 | 1.00 |
| TNF-α     | <0.01 | 4.4±1.8 | 4.18±1.6 | 3.79±1.9 | 2.66±2.2 | 1 | <0.01 | <0.01 | 0.14 | 0.60 | 0.93 | 0.65 |
| GM-CSF    | 0.92±0.2 | 0.7±0.21 | 0.45±0.1 | 1.28±1.1 | 0.38±0.2 | 0.91 | 0.13 | 0.71 | 0.58 | 0.96 | 0.11 | 1.0 |
| VEGF      | 98.31±59 | 77.22±54 | 77.1±77 | 15.86±18 | 26.18±22 | 1.0 | 0.94 | 0.05 | 0.41 | 0.81 | 1.0 | 0.73 |
| MCP-1     | 848±490 | 662±287 | 549±192 | 721±307 | 66.4±37 | 1.0 | 0.07 | 0.97 | <0.01 | 0.15 | 0.02 | 0.08 |
| ICAM-1*   | 314.9±87 | 430±87 | 1035±533 | 499±419 | 328±291 | 0.02 | <0.01 | 0.95 | 1.0 | 1.00 | 0.99 | 0.05 |

TC-term control; PC- preterm control; BPD- bronchopulmonary dysplasia; PH- pulmonary hypertension; BPD+PH- BPD associated pulmonary hypertension; IL: interleukin; TNF: tumor necrosis factor; GM-CSF: granulocyte macrophage-colony stimulating factor; VEGF: vascular endothelial growth factor; MCP: monocyte chemoattractant protein; ICAM: intercellular adhesion molecule; CLIC: chloride intracellular channel protein; ANG: angiopoietin.
**Table 2B**

Mean (±S.D.) levels of CLICs in different groups in discovery cohort; *levels in ng/ml

| Biomarker | TC          | PC          | BPD         | PH          | BPD+PH |
|-----------|-------------|-------------|-------------|-------------|--------|
|           | (pg/ml)     | (n=8)       | (n=8)       | (n=17)      | (n=7)  |
| CLIC 1*   | 16.9±3.6    | 17.4±7.5    | 13.7±6.2    | 18.1±4.7    | 12.6±9.3| 0.66   | 0.80 | 1.00 | 0.89 | 0.81 | 0.72 | >0.99 |
| CLIC 4    | 375±79      | 542±71      | 488±362     | 275±206     | 395±49  | 1      | 0.91 | 0.97 | >0.99 | 0.95 | 0.22 | 0.99 |

TC-term control; PC- preterm control; BPD- bronchopulmonary dysplasia; PH- pulmonary hypertension; BPD+PH- BPD associated pulmonary hypertension; IL: interleukin; ANG: angiopoietin.
Table 2C –
Mean (±S.D.) levels of ANG in different groups in discovery cohort

| Biomarker | TC       | PC       | BPD     | PH      | BPD+PH   | PC vs. BPD | TC vs. PH | TC vs. BPD+PH | PC vs. BPD+PH | PH vs. BPD+PH | BPD vs. BPD+PH |
|-----------|----------|----------|---------|---------|----------|------------|-----------|---------------|---------------|----------------|----------------|
| ANG 2     | (n=7)    | (n=5)    | (n=12)  | (n=5)   | (n=3)    | 1.003      | 0.47      | 0.99          | 0.17          | 0.29           | 0.04           |
| ANG 1     | 1902±808 | 718±249  | 937±493 | 1709±878| 2799±972 | 0.99       | 0.99      | 0.11          | <0.01         | 0.07           | <0.01          |

TC-term control; PC- preterm control; BPD- bronchopulmonary dysplasia; PH- pulmonary hypertension; BPD+PH- BPD associated pulmonary hypertension; IL: interleukin; ANG: angiopoietin.
Table 3:
Comparison of selected study cohort characteristics from the discovery and validation cohort, *Mean ± SD

| Variable            | PC (n=9) | BPD-SC (n=15) | BPD-NW (n=40) | PC vs. BPD-SC | PC vs. BPD-NW | BPD-SC vs. BPD-NW |
|---------------------|----------|---------------|---------------|---------------|---------------|--------------------|
| Birth weight* (g)   | 1175±191 | 739±270       | 809±228       | <0.01         | <0.01         | 0.69               |
| Gestational age* (wk)| 29.7±1.1 | 25.18±2.3     | 26.44±2       | <0.01         | <0.01         | 0.12               |
| Post Menstrual Age* (wk) | 36.6±1.4 | 36.8±1.6         | 36.8±0.6      | 0.95          | 0.97          | 0.99               |
| Gender- Female (n, %) | 8 (88.8) | 7 (46.6)       | 11 (27.5)     | 0.08          | <0.01         | 0.2                |

PC- preterm control from discovery cohort; BPD-SC- Moderate and Severe bronchopulmonary dysplasia from discovery cohort; BPD-NW- Moderate and Severe bronchopulmonary Dysplasia from validation cohort