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Early inflammatory mediator patterns in tracheal aspirate and the association with bronchopulmonary dysplasia (BPD) in low birth weight neonates

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Early inflammatory mediator patterns in tracheal aspirate and the association with bronchopulmonary dysplasia (BPD) in low birth weight neonates

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B.S., Emory University, 2006

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An abstract of
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Abstract

Early inflammatory mediator patterns in tracheal aspirate and the association with bronchopulmonary dysplasia (BPD) in low birth weight neonates
By Kari Schneibel

Despite advancements in the care of preterm neonates, the prevalence of bronchopulmonary dysplasia (BPD), a chronic lung disorder of neonates, in extremely premature infants has not decreased. While the etiology of BPD is thought to be multifactorial, studies have shown that alterations in pro- and anti-inflammatory mediators are an important modification in lungs of neonates who develop BPD. It is known that individual inflammatory mediators are involved in the development of BPD; however, there is a lack of current research examining the relationship between multiple inflammatory mediators in the lungs of premature neonates and the subsequent development of BPD. A pilot cross-sectional study was conducted in order to investigate whether the distribution of 12 inflammatory mediators detected in the tracheal aspirate (TA) of neonates within 24 hours of birth could differentiate between low birth weight neonates who did and did not develop BPD. TA samples were collected from 27 mechanically ventilated preterm neonates weighing less than 1,500 grams. BPD was diagnosed in 11 of the neonates at 36 weeks post-conceptional age. TA concentrations of IL-1ra, IL-1b, IL-4, IL-6, IL-8, IL-10, GM-CSF, VEGF, MCP-1, MIP-1a, MIP-1b, and TNF-alpha were determined by quantitative bead-based multiplex assay. There was no significant difference found between neonates who developed BPD and those who did not when individual levels of markers were compared. A linear discriminate analysis used to classify patients into those who did and those who did not develop BPD, based on the 12 measured biomarkers, displayed a significant level of discriminant function ($p=0.007$). While the levels of individual inflammatory mediators in low birth weight neonates at 24 hours may not differ between neonates who do and do not develop BPD, multiple inflammatory mediators can be used to classify neonates into who will and will not develop BPD.

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INTRODUCTION

The use of antenatal steroids, surfactant therapy, and gentler ventilation techniques have not only allowed an increasing number of smaller, extremely premature neonates to survive, but has also lead to a change in the pathophysiology of neonates who develop bronchopulmonary dysplasia (BPD) since severe lung injury in larger and more mature infants has been minimized (1). Significant morbidity in low birth weight neonates remains attributed to the development of BPD as it is the most common serious condition associated with premature birth (2).

BPD is characterized by an alteration or arrest of lung development, and is defined as supplemental oxygen requirement at 36 weeks post-conceptional age (3). Pulmonary inflammation plays an important role in the pathogenesis of BPD. Current research suggests that an imbalance between pro-inflammatory and anti-inflammatory mediators in the developing neonatal lung contributes to the development of BPD (4-6). The diagnosis of this condition in low birth weight neonates has been linked to the alteration of various interrelated biomarkers in the neonatal lung. Biomarker alterations indicated in the development of BPD include markers of poor endothelial integrity, increased fibrinolytic activity, increased oxidative stress, and abnormal lung repair (7).

A more in-depth understanding of the mechanisms controlling the inflammatory environment in the developing lung remains a priority for advances in the prevention, diagnosis, and treatment of BPD (1). Furthermore, early identification of the at-risk premature newborn would enable advances in patient-specific interventions to minimize the development of BPD.

While previous studies have examined the role of specific biomarkers in the lungs of neonates who develop BPD, little current research has studied the relationship between the levels of multiple inflammatory mediators in the tracheal aspirate (TA) of low birth weight infants prior to their development of BPD (8). The purpose of this study was to investigate whether the concentrations of IL-1ra, IL-1b, IL-4, IL-6, IL-8, IL-10, GM-CSF, VEGF, MCP-1, MIP-1a, MIP-1b, and TNF-alpha detected in the TA of neonates within 24 hours of birth could discriminate between low birth weight neonates who did and who did not develop BPD. We hypothesized there would be a different relationship among inflammatory mediators in the TA collected 24 hours postnatally of low birth weight neonates who developed BPD as compared to the inflammatory mediators in the TA of low birth weight neonates who did not develop BPD.

BACKGROUND

BPD was first described by Northway and colleagues in 1967 as a chronic respiratory disease seen in premature neonates exposed to mechanical ventilation and oxygen supplementation (9). However, with the use of antenatal corticosteroids, surfactant replacement, and a more conservative approach to respiratory support, there has been a substantial reduction in original form of lung injury described in BPD. This has lead to an improved survival rate among infants born at earlier gestational ages however, as a result, a new pattern of lung injury emerged. While the invent of new treatments improved the prevalence of the original form of BPD, the overall prevalence of BPD remained unchanged. In a recent study, 42% of extremely premature neonates in the study population developed BPD (11).

The new BPD is characterized by impaired alveolar septation, altered vascular growth, and bronchial mucosal metaplasia and hyperplasia (12). This new BPD has been described as an arrest in lung development, while old BPD was thought of as a structural lung injury (13). While the average age of neonate in Northway's original study was 33 weeks gestational age, neonates with the new BPD are 26 weeks gestational age on average at birth. At 26 weeks gestational age, neonatal lungs are between canalicular and saccular stage of lung development when alveolar ducts are just beginning to form (12). This is an earlier stage in the development of the lungs that was not disrupted in neonates with the old form of BPD. Furthermore, disturbance to the lung at this phase alters alveolar and vascular development (12).

There are multiple definitions of BPD, including a severity-based definition and a physiologic method based on the percentage of oxygen requirement and the resulting

oxygen saturation (1,14). However, the most accepted and utilized definition is a clinical diagnosis of supplemental oxygen dependency at 36 weeks postmenstrual age (3).

The etiology of BPD is thought to be multifactorial (10). While there are multiple risk factors for the development of BPD, it is still unclear which are the most important. It is thought that genetic susceptibility, antenatal exposures (steroids, chorioamnionitis, intrauterine growth restriction), and postnatal exposures (mechanical ventilation, supplemental oxygen, infection, steroids, pulmonary fluid overload, nutrional deficits) alter the risk of BPD in a neonate (13).

Pulmonary inflammation is a major contributor in the pathogenesis of BPD (15). BPD can be characterized by an imbalance between pro-inflammatory and anti-inflammatory mediators (4). Previous studies have focused on the role of specific cytokines and growth factors in the development of BPD (16-20). Biomarker alterations indicated in the development of BPD include markers of poor endothelial integrity, increased fibrinolytic activity, increased oxidative stress, and abnormal lung repair (7). Cytokines and growth factor alterations involved in the development of BPD, include IL-ra, IL-1b, IL-4, IL-6, IL-8, IL-10, GM-CSF, VEGF, MCP-1, MIP-1a, MIP-1b, and TNF-alpha (2,7,10,20,21).

Currently, providers are unable to clinically identify premature neonates who are at a higher risk of developing BPD early in the course of the disease. While it is known that individual inflammatory mediators are involved in the development of BPD, there is a lack of current research examining the relationship between multiple inflammatory mediators in premature neonates and the development of BPD. Furthermore,

inflammatory mediators are strongly interrelated leading to difficulty in teasing out the significance of individual mediators in the pathogenesis of this disease.

METHODS

Study Design. This pilot study assumed a cross-sectional design and was analyzed as a case-control study. TA samples collected and banked as part of a study examining neonatal alveolar macrophage maturation were utilized in this study.

Study Aims. The primary aim of this study was to investigate whether the distribution of the levels of 12 inflammatory mediators detected in the lungs of neonates within 24 hours of birth could differentiate between low birth weight neonates who did and did not develop BPD. The secondary aim was to examine whether the level of any individual inflammatory marker collected within 24 hours of birth differed among low birth weight neonates who developed BPD compared to those who did not.

Null Hypotheses. The null hypothesis for the primary aim was that relationship among the pulmonary inflammatory mediators collected 24 hours postnatally in low birth weight neonates who developed BPD would not differ from the relationship among the pulmonary inflammatory mediators of low birth weight neonates who did not develop BPD. The null hypothesis for the secondary aim was that the levels of each individual pulmonary inflammatory marker collected within 24 hours after birth in low birth weight neonates would not differ among those who did and did not develop BPD.

Subjects. After approval from the Emory IRB, subjects were enrolled from Emory Crawford Long Hospital and Grady Memorial Hospital in Atlanta, GA from November 2006-November 2008.

All neonates weighing less than 1,500 grams admitted to the neonatal ICU between the gestational ages of 23-31 weeks requiring intubation and mechanical ventilation were eligible for enrollment into the study. Premature newborns with

multiple congenital anomalies on physical exam were excluded due to the possible syndromic associations with macrophage function. Patients with clinically suspected or confirmed chromosomal abnormality were excluded for similar reasons. Neonates deemed non-viable by the attending neonatologist were not considered for enrollment. Patients with maternal HIV history were excluded because of the potential risk to laboratory personnel in the sample handling and fluid analysis. Finally, patients were excluded for maternal refusal to participate in the study.

Outcome variable. The outcome of the study was the diagnosis of BPD at 36 weeks gestational age by a health care provider. The diagnosis of BPD was determined by review of the patient medical record. BPD was defined as supplemental oxygen requirement at 36 weeks post conceptional age.

Predictor variables. A literature search was used to determine which biomarkers to include in the analysis of neonatal TA. Biomarkers found in TA that were linked to the development of BPD in previous studies were included. These markers were chosen for their biological relevance to BPD. The biomarkers measured in this study were IL-1ra, IL-1b, IL-4, IL-6, IL-8, IL-10, GM-CSF, VEGF, MCP-1, MIP-1a, MIP-1b, and TNF-alpha.

Procedures. After admission to the NICU and verbal consent from the mother, TA samples were collected during routine, clinically indicated ET tube suctioning by respiratory therapists within 24 hours of birth. For the suctioning procedure, bacteriostatic saline (~1 cc) was instilled into the trachea and after several ventilator breaths the sample was retrieved into a closed, sterile Leukins trap. The sample was immediately placed on ice and transported to the Gauthier laboratory for further analysis.

The samples were centrifuged at 1200 rpm for 8 minutes at 4°C to separate supernatant and cellular fractions. The supernatant was removed and divided into aliquots. Aliquots were stored at -80°C prior to analysis.

Concentrations of the cytokines IL-1ra, IL-1b, IL-4, IL-6, IL-8, IL-10, GM-CSF, VEGF, MCP-1, MIP-1a, MIP-1b, and TNF-alpha in the TA supernatant were determined using a quantitative bead-based Milliplex MAP Human multiplex assay (Millipore Corporation, Billerica, MA). The samples were ran with controls and serial dilutions of the standards and were added to 96-well plates. Plates were incubated overnight as per manufacture's protocol. Data were analyzed using a BioRad Bio-Plex System (Bio-Rad Laboratories, Hercules, CA) with gates of 4,335 and 10,000. The concentrations of the samples were determined using a 5-point logistic curve fitting algorithm (Bio-Plex Manager 3.0 Software; Bio-Rad Laboratories). Assay sensitivities for each biomarker are presented in Table I.

Sample Size Calculations & Statistical Analysis. As this was a pilot study, sample size calculations were not done prior to the start of the study. However, a post-study power analysis showed a power of 0.25, suggesting the appropriate power needed to detect a possible difference between the two groups using a t-test was not met. All data were analyzed with SPSS® for Windows software (Version 18.0, SPSS Inc., Chicago, IL). Patients were stratified to those who were diagnosed with BPD (BPD+) and those who were not diagnosed with BPD (BPD-). Raw biomarker concentrations were expressed in pg/mL and were logarithmically transformed prior to statistical analysis given their non-normal distribution (22). A significant difference was defined as $\alpha \leq 0.05$.

Gender and race were compared between the two groups using Fisher's exact test. Birth weight and gestational age were compared between the two groups using a chi-square test. In order to test the difference between the biomarker concentrations for each of the 12 biomarkers, the mean concentrations were compared using a student's T-test.

To examine the relationship between the 12 biomarkers measured and the diagnosis of BPD, a logistic regression analysis was performed with BPD (yes/no) being the outcome and the levels of IL-1ra, IL-1b, IL-4, IL-6, IL-8, IL-10, GM-CSF, VEGF, MCP-1, MIP-1a, MIP-1b, and TNF-alpha as predictors. A stepwise method, with an entry and removal probabilities of 0.05 and 0.10 respectively was used. Bivariate Pearson correlations were used to examine associations between the predictors.

Linear discriminant analysis using the Fisher method was used to classify patients into two outcome groups based on biomarker levels. LDA is a supervised learning method of classifying observations based on predictors. The model was generated from the linear combination of all 12 biomarkers measured in the TA fluid.

In order to determine a subset of predictors that could be used to classify patients into the two outcome groups, a logistic regression analysis using the stepwise method with entry and removal probabilities of 0.15 was completed. A linear discriminant analysis using the Fisher method was used to classify patients into two outcome groups based on the model generated from the linear combination of the 3 biomarkers found to be predictive of the outcome in this logistic regression analysis.

RESULTS

Characteristics of Study Population. The study included 11 neonates with BPD (BPD+) and 16 neonates without a diagnosis of BPD (BPD-). The total number of mothers approached for enrollment of their infant in the study was unknown. However, maternal refusal to participate was rare. Failure to obtain TA samples was a larger barrier to participation in the study.

The demographic and clinical characteristics of the 27 study neonates, shown in Table II, are grouped according to whether or not they were subsequently diagnosed with BPD. There was not a significant difference between the two groups when gestational age ($p = 0.584$), gender ($p = 0.130$), race ($p = 0.573$), and birth weight ($p = 0.377$) were compared.

Inflammatory Mediator Concentrations in TA. Table III displays the mean log transformed mediator concentrations obtained from analysis of the TA samples. The histograms for this data (not displayed) suggested that log transformation of the values did not completely normalize the data. However, this is common for cytokine data.

Comparison of Individual Biomarker Concentrations. Individual independent t-tests were used to compare the mean values of the 12 individual biomarker values between the BPD+ and BPD- groups (Table IV). There were no significant differences found between the two groups when individual levels were compared ($p > 0.05$ for all comparisons).

Linear Association of Biomarkers with Disease Outcome. To examine the association of the biomarker levels with BPD, a logistic regression analysis with the outcome of BPD (yes/no) and the 12 biomarkers as predictor variables was performed.

Using a stepwise method, with an entry and removal probability of 0.05 and 0.10 respectively, no predictors were included in the model.

Correlations between Biomarkers. A Pearson correlation matrix showed high correlation between most of the 12 biomarkers of pulmonary inflammation ($p < 0.05$) (Table V). The only biomarker pairs not significantly correlated were IL-4 and IL-8, IL-4 and MCP-1, IL-10 and IL-8, IL-10 and MCP-1, and MCP-1 and IL-4 ($p > 0.05$ for all comparisons).

Discrimination of Study Cases into BPD+ and BPD- Groups. A linear discriminate analysis (LDA) using the Fisher method was used to classify patients into two outcome groups (BPD+ and BPD-) based on the measured biomarkers. A function was generated from the linear combination of the 12 biomarker levels in order to calculate a discriminate score for each study case. The model displayed a significant level of discriminant function with a Wilk's Lambda = 0.24 and $p = 0.007$. To measure the strength of the relationship of the predictors to the outcome, the effect size was determined using the equation $\eta^2 = 1 - \lambda^{1/3}$. The effect size was determined to be 0.379, suggesting a large effect size.

Figure I displays the discriminant scores of all cases. The average discriminant score for patients without BPD was 1.42, while the average score for patients with BPD was -2.06. This graph illustrates the separation of the cases based on discriminant scores.

Classification of Study Cases Using the LDA Function. Using the 12-predictor model determined by LDA, the cases were classified based on the predictors (Table VI). A discriminate score was calculated for each subject using the function. Each subject was placed into a classification group based on which side of the cut point their score

placed them. 96% of all of the cases were correctly classified. All of the patients diagnosed with BPD were classified into the BPD+ group, meaning all BPD+ cases were correctly classified by the function. One of the BPD- cases was incorrectly classified as BPD+ and 15 of these cases were correctly classified as non-BPD cases.

Cross-validation classification was performed by classifying each case by a function, using 12 mediators as predictors, derived from all cases other than that case (Table VII). Analysis showed 81% of the cross-validated grouped cases were correctly classified. Of the patients who were diagnosed with BPD, 18.2% of the cases were misclassified. Of the patients who were not diagnosed with BPD, 18.8% of the cases were incorrectly classified as BPD+.

Determination of a Subset of Predictors. In order to establish a subset of predictors that could determine the outcome of BPD, a logistic regression was performed using a stepwise method with entry and removal probabilities of 0.15. The predictors included in the model were IL-4, IL-10, and TNF-alpha (Table VIII). IL-4 had the strongest relationship with the outcome ($p = 0.024$). A LDA using IL-4, IL-10, and TNF-alpha as predictors exhibited a significant level of discriminate function (Wilks' lambda = 0.691, $p = 0.035$).

Using the 3-predictor model determined by LDA, the cases were classified based on the predictors (Table IX). 77.8% of all cases were correctly classified. Among the patients who were not diagnosed with BPD, 25% were misclassified as BPD+. Of the patients who were diagnosed with BPD, 18.2% were misclassified as BPD-.

As with the 12-predictor model, cross-validation classification was performed by classifying each case by a function, using the mediators as predictors, derived from all

cases other than that case (Table X). The function correctly classified 71.4% of the cases when cross-validation was performed. Of the patients who developed BPD, 31.3% were misclassified. Of the patients who did not develop BPD, 27.3% of the patients were misclassified.

DISCUSSION

It is well known that pulmonary inflammation is associated with the development of BPD in premature infants (21). The association of BPD with pulmonary inflammation was first suggested with the observation of elevated neutrophil concentrations in the TA of neonates diagnosed with BPD (23). Research progressed to identify multiple pulmonary cytokine and growth factor alterations associated with the development of BPD (10,24).

The findings of this study were not in agreement with the results of previous studies that found differences between the levels of individual cytokines in the lungs of neonates who did and did not develop BPD (7). The results of this study do not suggest a difference between the group of patients who did and the group of patients who did not develop BPD when individual biomarker levels were compared. This was most likely due to the small sample size. This study was not adequately powered to find a difference between the two groups when individual biomarkers were compared.

The results of this study suggest that inflammatory mediators associated with BPD are highly related to one another as most of the mediators were highly correlated with the one another. These results further suggest that the development of BPD is linked to an alteration in the cytokine and growth factor milieu in the neonatal lung versus only isolated alterations in a few mediators.

This study was able to examine the development of BPD without potential postnatal exposures that occur after 24 hours of birth (ventilator use, supplemental oxygen use, infections, and nutritional deficits). This may suggest that antenatal exposures (steroids, chorioamnionitis, intrauterine growth restriction, and perinatal

inflammatory status) have a greater impact on the development of BPD than do postnatal exposures. Furthermore, these results suggest that early alterations in pulmonary expression of biomarkers are critical in the pathogenesis of BPD. This is in agreement with a prior study that found multiple growth factors were altered within 24 hours of birth in the lungs of neonates developing BPD (8).

The major limitation to this study was the small sampled size, which increased the probability of type II error. Additionally, we were not able to control for potential confounders such as chorioamnionitis or steroid use. We did not use a validation data set, so we were not able to fully assess the predictive value of the classification function developed. TA samples were collected at one point in time, while this is common in cross-sectional studies, we were not able to observe a pattern over time. However, even though levels were only examined at one early time point, the study has promising results as this one set of levels was able to discriminate between those who did and did not develop BPD.

Further study needs to be completed with a larger sample size in order to account for potential confounders. A subset of biomarkers that accurately classifies neonates who develop BPD and who do not needs to be identified. Additionally, a subset of biomarkers used to classify patients at multiple time points after birth needs to be examined. This would identify a time point where the association of biomarker levels with BPD is the greatest and potentially serve as tool to better predict the development of BPD early in the course of the disease.

Multiple inflammatory mediators in low birth weight neonates obtained within 24 hours of birth can be used to classify neonates into who will and will not develop BPD.

This study was able to determine a model for classifying patients' BPD status by biomarker levels. These findings may aid in identifying patients at a higher risk of developing BPD.

REFERENCES

1. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. *Am J Respir Crit Care Med*. 2001;163:1723-1729.
2. Bose CL, Dammann CE, Laughon MM. Bronchopulmonary dysplasia and inflammatory biomarkers in the premature neonate. *Arch Dis Child Fetal Neonatal Ed.* 2008;93:F455-F461.
3. Shennan AT, Dunn MS, Ohlsson A, et al. Abnormal pulmonary outcomes in premature infants: prediction from oxygen requirement in the neonatal period. *Pediatrics.* 1988;82:527–532.
4. Speer CP. Pre- and postnatal inflammatory mechanisms in chronic lung disease of preterm infants. *Paediatr Respir Rev*. 2004;5(suppl A):S241-2S44.
5. Speer CP. Pulmonary inflammation and bronchopulmonary dysplasia. *J Perinatol.* 2006; 26(suppl 1):557-562; discussion S63-64.
6. Speer CP. Inflammation and bronchopulmonary dysplasia: a continuing story. *Semin Fetal Neonatal Med.* 2006;11:354-362.
7. Thompson A, Bhandari V. Pulmonary Biomarkers of Bronchopulmonary Dysplasia. *Biomark Insights*. 2008;3:361-373.
8. Been JV, Debeer A, van Iwaarden JF, et al. Early alterations of growth factor patterns in bronchoalveolar lavage fluid from preterm infants developing bronchopulmonary dysplasia. *Pediatr Res.* 2010;67:83-89.
9. Northway WH, Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease: bronchopulmonary dysplasia. *N Engl J Med.* 1967;276:357-368.

10. Bhandari A, Bhandari V. Pitfalls, Problems, and progress in bronchopulmonary dysplasia. *Pediatrics*. 2009;123;1562-1573.

11. Stoll BJ, Hansen NI, Bell EF, et al. Neonatal outcomes of extremely preterm infants from the NICHD neonatal research network. *Pediatrics.* 2010;126(3):443–456.

12. Merritt T, Deming D, Boynton B. The 'new' bronchopulmonary dysplasia: challenges and commentary. *Semin Fetal Neonatal Med*. 2009;14(6):345-357.

13. Baraldi E, Filippone M. Chronic lung disease after premature birth. *N Engl J Med.* 2007;357:946-955.

14. Walsh MC, Yao Q, Gettner P, et al. Impact of physiologic definition on bronchopulmonary dysplasia rates. *Pediatrics.* 2004;114(5):1305-1311.

15. Pierce MR, Bancalari E. The role of inflammation in the pathogenesis of bronchopulmonary dysplasia. *Pediatr Pulmonol.* 1995;9:371-378.

16. Groneck P, Schmale J, Soditt V, et al. Bronchoalveolar inflammation following airway infection in preterm infants with chronic lung disease. *Pediatr Pulmonol.* 2001;31:331-338.

17. Baier R, Loggins J, Kruger TE. Increased Interleukin-8 and Monocyte Chemoattractant protein-1 concentrations in mechanically ventilated preterm infants with pulmonary hemorrhage. *Pediatr Pulmonol.* 2002;34:131-137.

18. Huang HC, Yang MY, Huang CB, et al. Profiles of inflammatory cytokines in bronchoalveolar lavage fluid from premature infants with respiratory distress disease. *J Microbiol Immunol Infect.* 2000;33:19-24.

19. Jones CA, Cayabyab RG, Kwong KY, 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. *Pediatr Res*. 1996;39(6):966-975.

20. Papoff P, Christensen RD, Calhoun DA, et al. Granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor and neutrophils in the bronchoalveolar lavage fluid of premature infants with respiratory distress syndrome. *Biol Neonate.* 2001;80(2):133-141.
21. Ryan RM, Ahmed Q, Lakshminrusimha S. Inflammatory mediators in the immunbiology of BPD. *Clin Rev Allergy Immunol*. 2008;34:174-190.
22. de Blic J, Midulla F, Barbato A, et al. Bronchoalveolar lavage in children. ERS Task Force on bronchoalveolar lavage in children. European Respiratory Society. *Eur Respir J.* 2000;15:217-231.
23. Merritt TA, Cochrane CG, Holcomb K. Elastase and alpha I-proteinase inhibitor activity in the tracheal aspirate during respiratory distress syndrome: role of inflammation in the pathogenesis of bronchopulmonary dysplasia. *J Clin Invest*. 1983;72:656-666.
24. Bhandari A and Bhandari V. Bronchopulmonary dysplasia: an update. *Indian J Pediatr*. 2007;74:73-77.

Table I. Assay sensitivities for inflammatory mediator levels detected by the Milliplex MAP Human multiplex assay.

Inflammatory Mediator	**Mean MinDC**[1]	**Mean MinDC + 2SD**[2]
IL-4	0.6	1.1
IL-6	0.3	0.7
IL-8	0.2	0.3
IL-10	0.3	0.5
GMCSF	9.5	18.9
TNF-alpha	0.1	0.1
IL-1b	0.4	0.7
MCP-1	0.9	1.6
MIP-1b	4.5	8.9
IL-1ra	2.9	5.5
MIP-1a	3.5	6.4
VEGF	5.8	10.3

[1] Mean minimum detectable concentration measured in pg/mL provided by Millipore Corporation.

[2] Mean minimum detectable concentration + 2 standard deviations measured in pg/mL provided by Miliipore Corporation.

Table II. Baseline characteristics of neonates who did (BPD+) and who did not (BPD-) develop bronchopulmonary dysplasia (BPD). Gender and race compared using Fisher's exact test. Gestational age and birth weight compared using Chi-square test. No significant difference found between BPD+ and BPD- groups. Data represent the mean ± SD or the frequency (%).

	BPD+ (n = 11)	**BPD-** (n = 16)	**P-value**
Gestational age (wks) mean±SD (minimum, maximum)	26.95±2.63 (24.0, 31.0)	26.18±1.86 (23.8, 29.0)	0.584
Gender number (%)			
Male	4 (36)	11 (69)	0.130
Female	7 (64)	5 (31)	
Race number (%)			
Caucasian	3 (27)	2 (13)	0.573
Black	7 (64)	13 (81)	
Hispanic	1 (9)	1 (6)	
Birthweight (g) mean±SD	817±263	872±245	0.377

Table III. Mean inflammatory mediator values for neonates who did (BPD+) and did not develop (BPD-) bronchopulmonary dysplasia (BPD). Data represent mean ± SD and are measured in pg/mL after log transformation.

Inflammatory Mediator	Mean±SD	
	BPD-	BPD+
IL-4	4.69±1.84	3.76±0.63
IL-6	8.67±1.48	8.24±2.29
IL-8	10.00±0.42	9.12±2.39
IL-10	5.06±2.10	5.04±1.73
GMCSF	6.07±1.42	5.36±0.88
TNF-alpha	6.39±2.33	6.18±2.14
IL-1b	6.36±2.34	6.31±2.18
MCP-1b	9.81±1.29	9.11±2.40
MIP-1b	7.15±2.56	7.15±2.32
IL-1ra	7.89±1.96	7.96±2.19
MIP-1a	7.93±2.58	7.68±2.37
VEGF	6.54±1.59	6.09±1.42

Table IV. Results of independent t-tests comparing individual biomarker values between the neonates who did and who did not develop bronchopulmonary dysplasia (BPD). T-value, 95% confidence interval, and p-value are presented for each comparison.

Inflammatory Marker	**T**	**95% CI**	**p-value**
IL-4	1.879	-0.10,1.97	0.075
IL-6	0.586	-1.07,1.92	0.563
IL-8	1.221	-0.73,2.51	0.249
IL-10	0.320	-1.55,1.61	0.975
GMCSF	1.460	-0.29,1.70	0.157
TNF-alpha	0.236	-1.61,2.03	0.815
IL-1b	0.063	-1.78,1.90	0.950
MCP-1	0.884	-1.00,2.40	0.392
MIP-1b	-0.004	-2.00,1.20	0.997
IL-1ra	-0.790	-1.72.1.59	0.938
MIP-1a	0.251	-1.77,2.26	0.804
VEGF	0.751	-0.78,1.68	0.460

Table V. Pearson correlation coefficients (*r*) for all 12 inflammatory biomarkers. (+) signifies a significant Pearson correlation coefficient between the two predictors ($p \leq 0.05$). Blank cells represent no significant correlation between the two predictors ($p > 0.05$).

	IL-4	IL-6	IL-8	IL-10	GMCSF	TNF-a	IL-1b	MCP1	MIP1b	IL-1ra	MIP1a	VEGF
IL-4		+		+	+	+	+		+	+	+	+
IL-6	+		+	+	+	+	+	+	+	+	+	+
IL-8		+			+	+	+	+	+	+	+	+
IL-10	+	+			+	+	+		+	+	+	+
GMCSF	+	+	+	+		+	+	+	+	+	+	+
TNF-a	+	+	+	+	+		+	+	+	+	+	+
IL-1b	+	+	+	+	+	+		+	+	+	+	+
MCP1		+	+		+	+	+		+	+	+	+
MIP1b	+	+	+	+	+	+	+	+		+	+	+
IL-1ra	+	+	+	+	+	+	+	+	+		+	+
MIP1a	+	+	+	+	+	+	+	+	+	+		+
VEGF	+	+	+	+	+	+	+	+	+	+	+	

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Table VI. Classification of study patients using 12-predictor model determined by LDA with bronchopulmonary dysplasia (BPD) status as the outcome. Actual group membership reflects the patient's BPD diagnosis (no BPD diagnosis = BPD-; diagnosed with BPD = BPD+). Represented as number of cases (% of study patients in corresponding BPD group). Yellow cells represent misclassified cases.

	Actual Group Membership	Group Membership Predicted by Function	
		BPD-	BPD+
Original	BPD -	15 (93.8%)	1 (6.3%)
	BPD+	0 (0%)	11 (100%)

Table VII . Cross-validation classification of study patients using 12-predictor models determined by LDA with bronchopulmonary dysplasia (BPD) status as the outcome. Cross-validation performed by classifying each case by a function, using all 12 mediators as predictors, derived from all cases other than that case. Actual group membership reflects the patient's BPD diagnosis (no BPD diagnosis = BPD-; diagnosed with BPD = BPD+). Represented as number of cases (% of study patients in corresponding BPD group). Yellow cells represent misclassified cases.

	Actual Group Membership	Group Membership Predicted by Function	
		BPD-	BPD+
Cross-validated	BPD-	13 (81.3%)	3 (18.8%)
	BPD+	2 (18.2%)	9 (81.8%)

Table VIII. Results of a logistic regression of the concentration of inflammatory biomarkers in tracheal aspirate (TA) on bronchopulmonary dysplasia (BPD) status modeled as yes=1, no=0.

Predictors	Coefficient	S.E.	Wald	Sig.
IL-4	-2.367	1.050	5.084	0.024
IL-10	2.330	1.234	3.567	0.059
TNF-a	-.950	0.654	2.112	0.146
Constant	3.799	2.329	2.661	0.103

Table IX. Classification of study patients using the 3-predictor (IL-4, IL-10, TNF-alpha) model determined by LDA with bronchopulmonary dysplasia (BPD) status as the outcome. Cross-validation performed by classifying each case by a function, using the 3 mediators as predictors, derived from all cases other than that case. Actual group membership reflects the patient's BPD diagnosis (no BPD diagnosis = BPD-; diagnosed with BPD = BPD+). Represented as number of cases (% of study patients in corresponding BPD group). Yellow cells represent the misclassified cases.

	Actual Group Membership	Group Membership Predicted by Function	
		BPD-	BPD+
Original	BPD -	12 (75%)	4 (25%)
	BPD+	2 (18.2%)	9 (81.8%)

Table X. Cross-validation classification of study patients using 3-predictor (IL-4, IL-10, TNF-alpha) models determined by LDA with bronchopulmonary dysplasia (BPD) status as the outcome. Actual group membership reflects the patient's BPD diagnosis (no BPD diagnosis = BPD-; diagnosed with BPD = BPD+). Represented as number of cases (% of study patients in corresponding BPD group). Yellow cells represent misclassified cases.

	Actual Group Membership	Group Membership Predicted by Function	
		BPD-	BPD+
Cross-validated	BPD -	11 (68.8%)	5 (31.3%)
	BPD+	3 (27.3%)	9 (72.7%)

Figure I. Discriminant score scatter plot displaying the discriminant score for each patient in the study. Each dot corresponds to a patient's discriminate score determined by the 12-predictor LDA function. On the X-axis the patients are grouped by actual bronchopulmonary dysplasia (BPD) diagnosis, which is plotted against their discriminative score on the Y-axis. The average discriminant score for patients without BPD (BPD-) was 1.42 and -2.06 for patients diagnosed with (BPD+).