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Prenatal Complications Are Associated with the Postnatal Airway Host Response and Microbiota in Intubated Pre-term Infants

Brandie D. Wagner, PhD¹, Marci K. Sontag, PhD², J. Kirk Harris, PhD³, Joshua I. Miller, MPH², Lindsey Morrow¹, Charles E. Robertson, PhD⁴, Mark J. Stephens, PhD³, Brenda B. Poindexter, MD, MS⁵, Steven H. Abman, MD^{3,7}, and Peter M. Mourani, MD^{6,7}

¹Departments of Biostatistics, Colorado School of Public Health, University of Colorado, Aurora, Colorado

²Departments of Epidemiology, Colorado School of Public Health, University of Colorado, Aurora, Colorado

³Department of Pediatrics, Section of Pulmonary Medicine, University of Colorado, Aurora, CO

⁴Department of Medicine, Section of Infectious Disease, University of Colorado, Aurora, CO

⁵Perinatal Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, OH.

⁶Pediatric Heart-Lung Center, Department of Pediatrics, University of Colorado, Aurora, CO

⁷Department of Pediatrics, Section of Critical Care Medicine, University of Colorado, Aurora, CO

Abstract

Purpose: To prospectively examine the relationship between prenatal events, postnatal airway host response and microbiota, and clinical outcomes.

Materials and Methods: Tracheal aspirates collected at 7 days of age from 71 mechanically ventilated infants (median gestational age (GA), 25 weeks [range 23–28] were simultaneously processed for a 12-plex protein assay and bacterial identification by 16S rRNA sequencing. Phenotypes were determined by unsupervised clustering of the protein analytes. Subject characteristics, microbial communities and clinical factors and outcomes were compared across the phenotype groups.

Results: Three clusters were identified: 1 (high protein levels), 2 (high pro-inflammatory proteins and low anti-inflammatory proteins) and 3 (low protein levels), respectively. Antenatal hemorrhage was most common in cluster 1, while Chorioamnionitis characterized cluster 2 and

Correspondance: Brandie D Wagner, PhD, 13001 East 17th Place, Box B119, Aurora, CO 80045, Tel: (303) 724-4478, brandie.wagner@ucdenver.edu.

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Contributions to the Study:

Conception and design: BDW, MKS, JKH, SHA, BBP, PMM

Acquisition of the data: JKH, JIM, MJS, BBP, PMM

Performed the analysis: BDW, MKS, JIM, LM, CER

Drafting the manuscript for important intellectual content: BDW, MKS, JKH, SHA, PMM

Review and revision of manuscript: BDW, MKS, JKH, JIM, LM, CER, BBP, SHA, PMM

Pre-eclampsia was most prevalent in cluster 3, which was characterized by a predominance of *Staphylococcus* and relative absence of *Ureaplasma*. There were higher rates of adverse clinical outcomes in cluster 1.

Conclusion: Airway protein profiles in 7 day old mechanically ventilated preterm infants are associated with important antenatal events and unique airway microbial communities. These relationships may reveal new mechanisms by which antenatal events impact the course and outcomes of preterm infants.

Keywords

preterm infants; airway microbiome; chorioamnionitis; antenatal hemorrhage; tracheal aspirate

Introduction

Although advances in perinatal medicine have increased survival of extremely premature newborns, preterm infants remain at high risk for late morbidity and mortality characterized by increased risk for infections, necrotizing enterocolitis, prolonged need for oxygen therapy, reactive airways and recurrent respiratory exacerbations with frequent hospitalizations.[1, 2] Early injury to the developing lung from prenatal and postnatal events can impair angiogenesis and alveolarization, which results in simplification of the distal lung airspace and clinical manifestations of bronchopulmonary dysplasia (BPD).[3, 4] However, the exact mechanisms that contribute to the pathogenesis remain poorly understood.

Previous studies have demonstrated that inflammatory responses to adverse *in utero* and *ex utero* stimuli, such as pre-eclampsia, chorioamnionitis, prolonged rupture of membranes, postnatal infections, mechanical ventilation, and oxygen therapy can disrupt lung growth after preterm birth.[5, 6] While infection has been a key inflammatory stimulus that can alter lung development, recent evidence indicates that bacterial exposures even without overt infection can modulate immune responses.[7] Likewise, immune responses may modulate the composition of host microbiota. Emerging data have revealed that normal lungs, previously considered a sterile microenvironment, contain diverse microbial populations, called microbiota. New molecular tools that detect bacterial DNA are now available and enable identification of bacterial communities in a specimen. Thus, investigations of the bidirectional interactions between airway microbes and inflammatory host responses and their impact on the outcomes of prematurely born infants are now possible.

In order to examine the relationship between the prenatal events, the respiratory tract microbiota and the host inflammatory response, we evaluated tracheal aspirates from mechanically ventilated preterm infants with a 12 protein multiplex assay and bacterial identification using the 16S rRNA gene. We performed cluster analysis based on the inflammatory protein markers to identify classes of infants with different host response profiles. We then assessed whether these classes were associated with clinical and microbiota characteristics.

Materials and Methods

Study design and subjects

The patient specimens and data for this study were obtained from a two-center observational longitudinal study that enrolled a cohort of preterm infants between July 2006 and February 2013. The protocol was approved by the Institutional Review Boards at each of the participating sites, and written informed consent was received from the parents or guardians of all participants. Criteria for enrollment included gestational age less than 34 weeks and birthweight between 500 and 1250 g. Exclusion criteria included clinical evidence of congenital heart disease (except patent ductus arteriosus, patent foramen ovale or atrial septal defect < 1cm, or ventricular septal defect < 2mm if known prior to enrollment); lethal congenital abnormality; and futile cases (anticipated death prior to hospital discharge). Subjects were required to be enrolled within 7 days of age.[8] Infants who were mechanically ventilated and had tracheal aspirates collected at 7 days $\pm$ 48 hours were included in this study, as these samples were collected closest to birth. Infants who died, withdrew, or were transferred or discharged prior to 36 weeks post menstrual age (PMA) were also excluded.

Detailed clinical data including maternal and birth history, hospital course and physiological assessments were collected. White blood cell (WBC) count if performed clinically at 7 days of age was recorded. Maternal antenatal factors for this study were collected from documentation in the mother's medical record as determined by her primary care team. Factors included antepartum hemorrhage (defined as significant vaginal bleeding prior to delivery), chorioamnionitis (defined as maternal fever, uterine tenderness, a foul-smelling vaginal discharge, rapid fetal heartbeat and maternal leukocytosis, or placental pathologic diagnosis, if available), gestational diabetes, pre-eclampsia (defined as systolic blood pressure $\geq$ 140 mm Hg or a diastolic blood pressure $\geq$ 90 mm Hg on two occasions at least 4 hours apart in a previously normotensive patient and a urine protein (mg/dL)/creatinine (mg/dL) ratio of $\geq$ 0.3 or a urine dipstick protein of 1+), prolonged rupture of membranes (defined as rupture of membranes $\geq$ 18 hours prior to delivery. Clinical outcomes included intraventricular hemorrhage (IVH defined as grade 3 or 4 by cranial ultrasound), necrotizing enterocolitis (diagnosed by the primary care team that includes the finding of pneumatosis on x-ray, both nonsurgical and surgical cases are included), retinopathy of prematurity (threshold retinopathy) and BPD status and severity (assessed at 36 weeks PMA using a modification of the NIH workshop definition[3] with application of the oxygen reduction test as described by Walsh[9] and described previously[8]).

Specimen collection and processing

Tracheal aspirate specimens were collected by installation of 0.5 mL of sterile 0.9% saline (without preservative) via the endotracheal tube followed by 3–5 ventilator breaths. Then a 5 or 6 French sterile suction catheter attached to a sterile specimen trap was inserted to 0.5cm below the tip of the endotracheal tube and secretions were suctioned as catheter was slowly withdrawn. Specimens were then centrifuged at 250g for 20 minutes at 4°C. The supernatant was utilized for a 12-plex high sensitivity protein assay (R&D Systems, Minneapolis, MN) and included IL-1 β , IL-2, IL-4, IL-5, IL-6, IL 8, IL-10, IL-12, granulocyte-macrophage

colony-stimulating factor (GM-CSF), interferon gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α), and vascular endothelial growth factor (VEGF) and the pellet containing the bacterial DNA was utilized for microbiota analysis.

Laboratory Assays

Quantitative PCR—DNA extractions were performed using the Qiagen EZ1 Advanced automated extraction platform (Qiagen Inc., Valencia, CA) with the bacterial card and tissue extraction kit. Details of the DNA extraction are available in the online supplement. Total bacterial load was measured using a quantitative real-time PCR assay that has been previously published.[10]

DNA sequencing—Bacterial profiles were determined by broad-range amplification and sequence analysis of 16S rRNA genes following our previously described methods.[11, 12] In brief, amplicons were generated using primers that target approximately 300 base pairs of the V1/V2 variable region of the 16S rRNA gene. *PCR* products were normalized using agarose gel densitometry, pooled, gel purified, and concentrated using a DNA Clean and Concentrator Kit (Zymo, Irvine, CA). Pooled amplicons was quantified using Qubit Fluorometer 2.0 (Invitrogen, Carlsbad, CA). The pool was diluted to 4nM and denatured with 0.2 N NaOH at room temperature. The denatured DNA was diluted to 20pM and spiked with 10% of the Illumina PhiX control DNA prior to loading the sequencer. Illumina paired-end sequencing was performed on the Miseq platform using a 500 cycle version 2 reagent kit.

Analysis of Illumina Paired-end Reads—As previously described, Illumina MiSeq paired-end sequences were sorted by sample via barcodes in the paired reads with a python script.[11] Sorted paired end sequence data were deposited in the NCBI Short Read Archive under accession number SRP066782. Operational taxonomic units (OTUs) were produced by clustering sequences with identical taxonomic assignments. This process generated 4,797,688 sequences for 71 samples (average size: 67,573 sequences/sample; min: 8,852; max: 196,691). The software package Explicet (v2.10.5, www.explicet.org)[13] was used to calculate rarefied Good's coverage and alpha diversity measures.

Statistical Analyses—Simple correlations between protein biomarkers and the prominent taxa were estimated using Spearman's rank based correlation coefficients. Unsupervised clustering using the RandomForest package in R (version 3.3.1) of the 12 protein analytes was performed and consisted of 5,000 trees (all other default parameters were used).[14] Partitioning around medoids clustering on the resulting proximity matrix was used to determine phenotypes. Subject characteristics, microbial communities, clinical factors and outcomes were compared across the phenotype groups using chi-squared tests and Wilcoxon based tests for categorical and continuous variables, respectively. To account for differences in sequencing depth, the relative abundance (RA) of each taxon was calculated (number of sequences for specific taxa/ total number of sequences*100).

Results

There were 425 infants enrolled in the parent study. Of 333 infants requiring mechanical ventilation, 201 had at least one tracheal aspirate collected, and 74 of these had one specimen collected between days 5–9 that amplified sufficiently for microbiota analysis and was successfully processed for proteomic analysis. All but 3 of these subjects developed BPD, further description of these subjects is included in the online supplement. The characteristics of the study cohort are listed in Table 1. Samples were collected at a median of 7 days of life. The median gestational age at birth was 25 weeks and ranged from 23–28 weeks.

Description of Inflammatory Protein Phenotypes

Correlation coefficients between protein biomarkers and the prominent taxa revealed patterns of association between groups of proteins and dominance of specimens with *Staphylococcus*, *Streptococcus*, and *Gemella* (Figure E1 in the online supplement). Based on these results, we performed an unsupervised random forest (RF) of the 12-plex protein assay values which identified three phenotype clusters (Figure E2), with 24, 23, and 24 infants classified into clusters 1, 2 and 3, respectively. Cluster 1 had high levels for almost all the proteins measured, whereas Cluster 3 is marked by very low levels of all of the proteins (IL-1 β , IL-2, IL-5, IL-6, IL-8, IL-10, IL-12, TNF- α , VEGF, GM-CSF, and IFN- γ), and Cluster 2 had a mix of high levels of proteins that are generally characterized as pro-inflammatory (GM-CSF, IL-1 β , IL-6, IL-8, TNF- α and VEGF) and lower levels of proteins that are generally characterized as anti-inflammatory (IFN- γ , IL-2, IL-4, IL-10, IL-12). Individual proteins are displayed across each of the three clusters in Figure E2b and Table E1 in the online supplement. Infants in Cluster 2 also had the highest WBC at 7 days of age (Table 1).

Description of Clinical Characteristics Across Phenotypes

In order to assess features that may be associated with each phenotypic inflammatory protein cluster, we examined antenatal and early postnatal factors, the microbiota from the tracheal aspirates, and clinical outcomes from each neonate. Maternal antenatal factors were different across the three groups (Table 1). Antenatal hemorrhage was most common in Cluster 1 (29%) compared to Cluster 2 (4%) and Cluster 3 (0), $p < 0.01$. Cluster 2 had higher frequencies of chorioamnionitis (35% compared to 4% in Cluster 1 and 8% in Cluster 3; $p = 0.02$) and prolonged rupture of membranes (35% compared to 8% in both Cluster 1 and Cluster 3; $p = 0.03$). Pre-eclampsia was most prevalent in Cluster 3 (42% compared to 21% in Cluster 1 and 9% in Cluster 2), but was not statistically different than the other groups. There were no differences in other antenatal or early postnatal factors (multiple gestation, delivery by cesarean section, gestational age, birth weight z-score). There was no difference in whether infants had received iNO treatment or in FiO₂ at day 7 but mode of O₂ delivery showed marginal differences across clusters, with cluster 1 having the highest rate of conventional ventilation, cluster 2 with the highest rate of high frequency oscillator ventilator and cluster 3 with the highest rate for CPAP.

The median total bacterial load for the cohort was 4.8 (IQR, 3.6 – 7.5) log₁₀ copies/reaction, and there was no difference between the Clusters (Table 1). The subject level microbial communities are displayed across the three phenotype groups in Figure 1a. The median (range) of Shannon diversity and Evenness were 0.1 (0.01 – 2.23) and 0.03 (0.004 – 0.52) for the entire cohort. Cluster 3 exhibited the lowest median Diversity (Figure 1b) and Evenness (Figure 1c) values, but these were not statistically significant. Investigating individual microbial community profiles across the three Clusters illustrates that *Staphylococcus* was most dominant in Cluster 3 (median relative abundance of 99.2% compared to 94.8% in Cluster 1 and 94.7% in Cluster 2; p-value =0.03; Figure 1d and Table E2). Cluster 3 also had relative absence of *Ureaplasma* with almost no subjects exhibiting a relative abundance greater than 1%, although the difference in median relative abundance for the group was not statistically significant (0.033% compared to 0.038% in Cluster 1 and 0.47% in Cluster 2; Figure 1e and Table E2). *Streptococcus* was most abundant in Cluster 1 (median relative abundance of 0.13% compared to 0.013% in Cluster 2 and 0.008% in Cluster 3; p-value <0.01; Table E2). *Gemella* was also found to be statistically most abundant in Cluster 1, but all Clusters exhibited extremely low relative abundance of this taxon.

Although no one outcome was statistically significant, Cluster 1 had higher rates of moderate/severe BPD, retinopathy of prematurity (ROP), and proportion of subjects discharged on oxygen, and longer total days on oxygen and ventilation days (Table 1). Clusters 2 and 3 had similar lung related outcomes, but Cluster 2 had lower rates of IVH, ROP, sepsis and necrotizing enterocolitis compared to Cluster 3. Only 2 deaths occurred, both subjects were in cluster 2.

Discussion

We evaluated the host airway response for 12 proteins in tracheal aspirates collected at 7 days of age from preterm infants requiring mechanical ventilator support. We found that the infants segregated into 3 Clusters based on the profile of these proteins. One cluster was characterized by high levels of most proteins, the second by a mix of high levels of pro-inflammatory proteins and low levels of anti-inflammatory proteins, and the third by low levels of all proteins (Table E1 and Figure E2b). We identified an association between these biomarker clusters and antenatal factors, such that infants exposed to antepartum hemorrhage were more likely to exhibit a Cluster 1 profile, those exposed to chorioamnionitis or prolonged rupture of membranes were more likely to exhibit a Cluster 2 protein profile, and those exposed to pre-eclampsia were more likely to exhibit Cluster 3 phenotype. Further, there were subtle differences in the airway microbiota profile among these biomarker clusters, such that Cluster 3 infants were more likely to be dominated by *Staphylococcus* with relative absence of *Ureaplasma* and Cluster 1 exhibited the highest levels of *Streptococcus* and *Gemella*. Thus, there appears to be important associations between antenatal events, the host airway immune response and airway microbiota (Table 2).

These associations may demonstrate the critical influence of antenatal exposures on the immune response and acquisition of the airway microbiota after birth in premature infants. Interestingly, infants exposed to frank infection (chorioamnionitis) or early environmental

exposure (prolonged rupture of membranes) exhibited a profile with higher pro-inflammatory cytokines (IL-1 β , IL-6, IL-8, TNF- α), low levels of anti-inflammatory cytokines (IFN- γ , IL-10, IL-12), and had the highest WBC. Inflammation has been an important factor in the pathophysiology of lung growth impairment and believed to result from multiple etiologies, including pre- and postnatal infection, and intrauterine growth restriction.[15, 16, 17] We found that infants exposed to clinical chorioamnionitis had higher levels of inflammatory markers in their airways at 7 days of age. These data are consistent with previous studies that identified higher levels of these same markers in the lower airways of infants exposed to clinical and histological chorioamnionitis.[18, 19, 20]

Further, Cluster 3, which had the lowest protein levels of the cohort had higher rates of infants from mothers with pre-eclampsia. Higher cytokine levels have been reported in pre-eclamptic maternal plasma samples [21], however, maternal pre-eclampsia can result in neonatal thrombocytopenia and platelet production is influenced by cytokines and growth factors [22] which may explain the association between pre-eclampsia and lower cytokine levels found in our infant airway samples. This cluster also tended to be dominated by *Staphylococcus*, and was markedly lacking in *Ureaplasma*. It is possible that *Staphylococcus* species may serve as commensal organisms that do not elicit a significant inflammatory response. Likewise, these data support previous reports that *Ureaplasma* can play a role in inducing airway inflammation.[23, 24, 25] Alternatively, it is also possible that different host immune response lead to acquisition of different colonizing microbes. *Streptococcus* and *Gemella* were in low abundance in all groups, but Cluster 1 exhibited statistically significant higher relative abundance for these microbes compared to Clusters 2 and 3. Given the low relative abundance of these organisms in all groups, it is not clear whether the differences have biologic importance. However, one small study found that overall low relative abundance of Lactobacillus in the airways of preterm infants shortly after birth, infants who went on to develop BPD had statistically lower relative abundance of this genus than infants who did not develop BPD.[26] Previous studies have associated specific microbiota patterns with increased inflammation in other human systems. The acquisition and composition in the intestinal tract microbiota of adults and premature infants has been associated with inflammation and altered epithelial barrier function.[27, 28]

Previous evaluations of airway cytokines have found associations with development of BPD. [18, 20] An association between perinatal inflammation and ROP has also been shown,[29] although it is curious that the association was seen here with inflammation measured in airway samples. Infants in Cluster 2 defined by higher pro-inflammatory and lower anti-inflammatory markers had higher exposure rates to chorioamnionitis or prolonged rupture of membranes, as well as lower morbidity outcomes for both lung and non-lung related outcomes. It is possible that the immune priming in these patients may improve the ability to withstand early NICU morbidities, further studies would be needed to determine longer term morbidities. Alternatively, these antenatal factors are also more likely to result in prolonged monitoring during pregnancy and possible interventions (i.e. steroids and antibiotics) which have been shown to improve neonatal outcomes[30, 31, 32], although in our data we saw no difference in maternal steroids or antibiotics across the clusters. A recent study found that prolonged rupture of membranes had lower rates of ROP which decreased further if the

rupture was greater than 24 hours prior to birth providing some evidence for this speculation. [33]

There are limitations to this study, first, because analysis of the airway microbiota requires access to the lower airway with an endotracheal tube, the airway microbiota in infants who did not require mechanical ventilation was not included. As such, we did not have sufficient numbers of infants who were mechanically ventilated that did not go on to develop BPD, hindering our ability to determine whether these protein profile clusters could differentiate between those with and without BPD. Second, the diagnosis of chorioamnionitis for this study was a clinical diagnosis made by the caring physician and was not confirmed by histological examination, thus there could be false positives and false negatives for this outcome. Thirdly, 16s rRNA sequencing as employed for our study, did not achieve the resolution to definitively identify bacteria at the species level. Thus, it is difficult to ascertain what proportion of some bacteria identified at the genus level (e.g. *Staphylococcus*) represent commensal vs. pathogenic species. Finally, these associations do not prove a causal relationship between antenatal factors and the early postnatal host response or microbiota acquisition, but they do present compelling avenues of further investigation to identify mechanisms in the pathogenesis of BPD and other morbidities associated with preterm birth.

In conclusion, we found the airway protein profile in one week old mechanically ventilated preterm infants is associated with important antenatal factors, including chorioamnionitis, prolonged rupture of membranes, antenatal hemorrhage, and possibly pre-eclampsia, and may be associated with unique airway microbiota patterns. Further investigation of these relationships may elucidate new mechanisms of lung development after preterm birth and identify novel targets to enhance lung development and prevent or mitigate respiratory disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BPD	Bronchopulmonary dysplasia
GM-CSF	granulocyte-macrophage colony-stimulating factor
IFN-γ	interferon gamma
IL	interleukin

PMA	postmenstrual age
RF	random forest
TNF-α	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor
WBC	white blood cell

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Figure 1:

(A) Subject level microbial communities are displayed across the three phenotype groups. Bacteria are identified at the genus level. (B) Shannon diversity of the airway bacterial communities across the 3 clusters revealed Cluster 3 to have the lowest diversity, but this difference is not statistically significant. (C) Cluster 3 also exhibited the lowest median Evenness values, but the difference is not statistically significant. (D) *Staphylococcus* was most dominant bacteria in Cluster 3 (median relative abundance of 99.2% compared to 94.8% in Cluster 1 and 94.7% in Cluster 2; p -value = 0.03). (E) Cluster 3 also had relative

absence *Ureaplasma* with almost no subjects exhibiting a relative abundance greater than 1%, although the for the group was not statistically significant (0.033% compared to 0.038% in Cluster 1 and 0.47% in Cluster 2).

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Table 1:

Clinical, prenatal characteristics and clinical outcomes across three clusters identified through random forest unsupervised clustering.

Variable	All patients (n = 71)	Cluster 1 (N = 24)	Cluster 2 (N = 23)	Cluster 3 (N = 24)	p-value
Gender (male)	41 (58%)	14 (58%)	16 (70%)	11 (46%)	0.28
Day of collection of tracheal aspirate	7 (5 – 9)	7 (6 – 9)	7 (5 – 9)	7 (5 – 9)	0.03
Birth weight z-score	–0.01 (–1.37 – 1.31)	0.04 (–1.25 – 0.72)	0.04 (–1.25 – 1.10)	–0.09 (–1.37 – 1.31)	0.89
Gestational age at birth (wks)	25 (23 – 28)	25 (23 – 28)	25 (24 – 28)	25 (23 – 28)	0.89
Antenatal Steroids (n=69)	59 (86%)	19 (79%)	18 (78%)	22 (92%)	0.74
Antenatal Antibiotics (n=64)	47 (73%)	16 (73%)	17 (77%)	14 (70%)	0.94
Antepartum hemorrhage (n=67)	8 (12%)	7 (29%)	1 (4%)	0	<0.01
Chorioamnionitis (n=56)	11 (20%)	1 (4%)	8 (35%)	2 (8%)	0.02
Gestational Diabetes (n=63)	4 (6%)	2 (8%)	1 (5%)	1 (4%)	0.96
Pre-eclampsia (n=64)	17 (27%)	5 (21%)	2 (9%)	10 (42%)	0.10
Prolonged rupture of membranes	12 (17%)	2 (8%)	8 (35%)	2 (8%)	0.03
C-section (n=49)	36 (73%)	11 (85%)	12 (75%)	13 (65%)	0.43
Multiple gestation	12 (17%)	5 (12%)	5 (22%)	2 (8%)	0.42
FiO ₂ at day 7	0.30 (0.21 – 0.95)	0.30 (0.23 – 0.65)	0.32 (0.21 – 0.95)	0.30 (0.21 – 0.60)	0.25
mode					0.09
Conventional ventilation	54 (76%)	21 (88%)	15 (65%)	18 (75%)	
High frequency oscillator ventilator	9 (13%)	2 (8%)	6 (26%)	1 (4%)	
Continuous positive airway pressure	8 (11%)	1 (4%)	2 (9%)	5 (21%)	
iNO treatment at day 7 (n = 57)	3 (5%)	1 (4%)	0	2 (11%)	0.47
Alpha diversity (Shannon H)	0.1 (0.01 – 2.23)	0.1 (0.03 – 1.37)	0.13 (0.01 – 1.36)	0.09 (0.01 – 2.23)	0.39
Evenness (Shannon E)	0.03 (0.004 – 0.52)	0.03 (0.01 – 0.35)	0.05 (0 – 0.43)	0.02 (0 – 0.52)	0.36
Total bacterial load, log10 copies/reaction	4.8 (3.6 – 7.5)	5 (3.7 – 7.5)	4.6 (3.6 – 7.5)	4.4 (3.7 – 6.7)	0.63
White Blood Cell Count (10 ³ cell/mcl):	15.1 (3.8 – 50.5)	14.5 (3.8 – 31.1)	20.0 (6.5 – 50.5)	12.7 (4.3 – 43.5)	0.03
Clinical Outcomes					
Moderate or Severe BPD		21 (88%)	15 (65%)	15 (63%)	0.11
Death prior to discharge		0	2 (9%)	0	0.10
Discharged/transferred on oxygen (n=70)		22 (92%)	16 (70%)	20 (83%)	0.14
Total days of mechanical ventilation		41 (3 – 236)	27 (7 – 72)	26 (2 – 278)	0.26
Total days on CPAP		59 (21 – 271)	46 (16 – 128)	45 (6 – 304)	0.19

Variable	All patients	Cluster 1	Cluster 2	Cluster 3	p-value
	(n = 71)	(N = 24)	(N = 23)	(N = 24)	
Total days of oxygen		120 (42 – 288)	100 (43 – 181)	108 (59 – 340)	0.14
Postnatal steroids at any time		21 (88%)	16 (70%)	16 (67%)	0.20
IVH		1 (4.2%)	1 (4.4%)	2 (8.3%)	0.99
ROP		10 (42%)	3 (13%)	5 (21%)	0.06
Sepsis		8 (33%)	7 (30%)	9 (38%)	0.88
NEC		5 (21%)	2 (9%)	5 (21%)	0.49
Surgically treated NEC		5 (21%)	0	3 (13%)	0.09

N (percent) or median (range) presented. BPD: bronchopulmonary dysplasia; CPAP: continuous positive airway pressure; IVH: intraventricular hemorrhage; NEC: necrotizing enterocolitis; ROP: retinopathy of prematurity.

Table 2.
Clusters: Relationship Between Host Response, Antenatal Stress, and Airway Microbiota

Cluster	Protein Analytes		Antenatal Stress		Airway Microbiota		
	Pro-Inflammatory	Anti-Inflammatory	Antepartum Hemorrhage	Chorioamnionitis and PROM	Pre-eclampsia	Relative Abundance of Taxa	Evenness
						<i>Staph.</i>	<i>Ureapl.</i>
1	↑	↑	++	+	+	++	+
2	↑	↓	-	+++	-	++	+
3	↓	↓	-	+	+++	+++	-

PROM: premature rupture of membranes; *Staph.* *Staphylococcus*; *Ureapl.* *Ureaplasma*.