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PULMONARY HYPERTENSION IN THE PREMATURE INFANT POPULATION: ANALYSIS OF ECHOCARDIOGRAPHIC FINDINGS AND BIOMARKERS

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Abstract

Objective—Extremely low gestational age neonates (ELGANs) are at risk for pulmonary hypertension (PH). We hypothesized that PH, defined by echocardiogram at 36 weeks gestational age (GA), would associate with respiratory morbidity, increased oxidant stress, and reduced nitric oxide production.

Study Design—ELGANs in the Vanderbilt fraction of the Prematurity and Respiratory Outcomes Program (PROP) who had echocardiograms at 36 ± 1 weeks GA were studied. Echocardiogram features of PH were compared with clinical characteristics as well as markers of oxidant stress and components of the nitric oxide pathway. Biomarkers were obtained at enrollment (median day 3), 7, 14, and 28 days of life.

Results—Sixty of 172 infants had an echocardiogram at 36 weeks; 11 had evidence of PH. Infants did not differ by PH status in regards to demographics, respiratory morbidity, or oxidant stress. However, odds of more severe PH were significantly higher in infants with higher nitric oxide metabolites (NOx) at enrollment and with a lower citrulline level at day 7.

Conclusions—Respiratory morbidity may not always associate with PH at 36 weeks among ELGANs. However, components of nitric oxide metabolism are potential biologic markers of PH in need of further study.

Keywords

Pulmonary Hypertension; Bronchopulmonary Dysplasia; Biomarkers

Introduction

Extremely-low gestational age newborns (ELGANs; infants born between 23 and 29 weeks gestational age) represent a particularly vulnerable population. These infants have many clinical challenges and with increased survival, diagnosis and management of comorbidities, such as pulmonary hypertension (PH), is becoming increasingly important (1). In premature infants, the increased incidence of PH is likely multifactorial, including altered lung development, with decreased alveolarization and reduced pulmonary capillary surface area with subsequent PH (2, 3). PH describes a syndrome of hypertensive pulmonary vascular disease, typically characterized by a mean pulmonary artery pressure which exceeds 25 mmHg (4). Cardiac catheterization remains the gold standard for diagnosis of PH, but current diagnostics rely heavily on non-invasive assessment by echocardiogram due to the high risk nature of this invasive study in this vulnerable population. Supportive treatments include reducing hypoxemia, nutritional support, and reducing secondary insults to the developing lungs. Beyond supportive care, there is a role for PH-specific pharmacotherapy in the right clinical situation (5, 6), though continued research is needed.

As the population of ELGANs with PH grows, there is a need to better characterize the phenotype of infants with this comorbidity so that earlier identification and intervention can occur. Current evidence indicates that a diagnosis of bronchopulmonary dysplasia (BPD) associates with increased risk of developing PH (7) and that these infants have increased length of stay in the neonatal intensive care unit (NICU) (8). There is also a need for early biomarkers that predict the development of PH, as such biomarkers have the potential to provide time for intervention prior to PH development.

The Prematurity and Respiratory Outcomes Program (PROP) was a prospective multi-center observational trial of premature infants born between 23 and 29 weeks gestational age (9–11). Clinical parameters as well as biological samples were collected from the infants in a prospective manner with the main objective being to better understand long-term respiratory morbidity in this population. The PROP cohort presents a unique opportunity to study a well phenotyped cohort of ELGANs followed over time.

This study is a retrospective analysis of features of PH determined from clinical echocardiograms obtained at 36 weeks gestational age from the infants enrolled in PROP at our center. These echocardiographic features of PH, determined by a blinded external review of each study, were then associated with clinical parameters and biologic markers that were prospectively collected during the PROP study.

Methods

Study Design

The Improving Prematurity-Related Respiratory Outcomes at Vanderbilt (IMPROV) (U01HL101456) was a prospective observational study that was part of the multi-center Prematurity and Respiratory Outcomes Program (PROP; U01 HL101794) (9). The main objective of this study was to evaluate longitudinal respiratory outcomes, up to 1 year corrected age, for extremely low gestational infants (ELGANS) in an effort to better understand the mechanistic and therapeutic targets of neonatal lung disease in this population. For the IMPROV cohort, in addition to the prospective collection of clinical data, biological samples were also collected four times in the first month of life for biomarker analysis and association with clinical parameters. The study was approved by the Vanderbilt IRB (#110833) and is listed in clinicaltrials.gov (NCT 01435187). All infants in this study were enrolled at Vanderbilt. The study protocol, including a review of information that would be collected, was discussed with parents of qualifying infants who met the inclusion and exclusion criteria. Informed consent was obtained from at least one parent.

For this study, echocardiographic images were obtained from the Xcelera database, which is part of the medical record. These images were obtained from infants enrolled in IMPROV who had echocardiograms obtained for clinical purposes at 36 weeks corrected gestational age ($\pm$ 1 week). There were no study-specific criteria for obtaining the echocardiograms. Echocardiograms were blindly read by a single pediatric cardiologist, specifically evaluating the images for features of PH and other irregularities, as outlined in table 1. In general, there was agreement between the blinded echocardiogram read and the clinical read.

Echocardiographic features were then compared to clinical features, including measures of respiratory morbidity, in addition to specific biochemical markers, as described below, that were obtained prospectively in the first month of life as part of the IMPROV protocol.

Participants

Full inclusion and exclusion criteria for PROP have been previously described (9). Briefly inclusion criteria included a gestational age between 23 0/7 and 28 6/7 weeks of gestation and less than 7 days of age at time of enrollment. Exclusion criteria included structurally significant congenital heart disease (not including patent ductus arteriosus and hemodynamically insignificant ventricular septal defect or atrial septal defect), structural abnormalities of the lung, and congenital malformations or syndromes expected to adversely affect life expectancy.

Clinical Endpoints

Clinical markers of respiratory status while in the NICU included days on mechanical ventilation, days on non-invasive ventilation (not intubated), and days with supplemental oxygen support by nasal cannula. These markers were obtained from the IMPROV database as well as chart review of the individual participants. In addition, birth weight, gestational age, weight gain per day, and length of stay in the NICU were gathered from the prospective IMPROV clinical database. Additional measures of respiratory status included: BPD defined as failing a room-air (RA) challenge at 36 weeks gestational age as previously described (9),

and postprematurity respiratory disease (PRD) at one year as defined by Keller et al (11). All biomarker data were stored in a REDCap (research electronic data capture) database.

Biochemical Markers

Samples for all plasma and urinary biochemical markers were obtained at time of enrollment (median of 3 days of life), 7 days, 14 days, and 28 days of life (± 2 days). Selected markers of inflammation included plasma isoprostane and isofuran levels. These markers specifically represent oxidant stress, as they are byproducts of lipid peroxidation (12). Isoprostane and isofuran levels were determined in the Vanderbilt Eicosanoid Core Laboratory by methods previously described (12).

Nitric oxide metabolites (NO_x), specifically nitrite and nitrate, were analyzed from urine specimens and corrected for urine creatinine. Urine NO_x concentrations were measured by chemiluminescence analysis as previously described (13). In brief, urine samples were diluted 1:9 with water and injected (20 μ l) into the reaction chamber of a chemiluminescent NO analyzer (model 170B NOA, Sievers). The reaction chamber contained vanadium (III) chloride in 1 M HCl heated to 90°C to reduce nitrite and nitrate to NO gas. The NO gas was carried into the analyzer using a constant flow of N₂ gas via a gas bubble trap containing 1 M NaOH to remove HCl vapor. A standard curve was generated by adding known amounts of NaNO₃ to distilled water and assaying as described for the urine samples.

Plasma amino acid profiles were quantified in Vanderbilt's clinical chemistry laboratory using a standard CLIA-approved method on a Hitachi 8800 amino acid analyzer. Amino acid levels were measured in batches using a plasma sample of 30 μ l.

Statistical Considerations

Continuous variables were summarized using the median with upper and lower quartiles and categorical variables were summarized using percentages. Changes in biomarkers over time were visualized using spaghetti plots where repeated measurements on each subject are connected with black lines and average trend shown with a thick red line. One year PRD was coded as a dichotomous outcome, and PH status was treated as either a 3-level ordinal outcome in regression models or a binary outcome (present/not present) in descriptive tables. Statistical tests by PH presence were done using the Wilcoxon signed-rank test for continuous variables or Pearson's chi-squared test for categorical variables. Proportional odds ordinal logistic regression was used to estimate odds ratios for the association of biochemical makers with PH severity. Separate models were fit for each biomarker at each time point. Due to limited number of subjects with PH, we did not estimate adjusted models. All odds ratio are expressed relative to a doubling of the level of the respective biochemical marker with an upper and lower 95% confidence intervals and corresponding p-value. We present the results of all estimated odds ratios and do not make any formal corrections for multiple comparisons.

Analyses were conducted using the R statistical program version 3.3.0 following principles for reproducible research.

Results

Echocardiograms

Of the 172 infants alive at 36 weeks GA and enrolled in IMPROV at the Vanderbilt site, 60 infants had an echocardiogram at 36 weeks $\pm$ 1 week. Of these 60 infants, 11 had an echocardiogram with features of PH (supplement figure 1). Degree of PH was categorized as mild, moderate, and severe based on estimated right ventricular pressure (RVP) by echocardiographic indices including tricuspid regurgitation jet, septal position and left ventricular systolic circular index (LVSCI). Mild PH correlates to about half systemic RVP, moderate is half to three-fourth systemic RVP and severe is systemic RVP. Seven infants were classified as having mild PH and 4 infants classified as having moderate PH (table 1). No infants were classified as having severe PH. Due to lack of complete envelope of the tricuspid regurgitation (TR) jet in all cases in which TR was present, classification of severity of PH was mostly based on the geometry of the interventricular septum.

Participants and Clinical Features

The basic demographics and clinical features for the 60 infants who had an echocardiogram at 36 weeks corrected GA did not reveal any statistical differences for gestational age, gender, or birth weight when the infants were divided by PH status (Table 2). In terms of respiratory morbidity, there were no differences in days spent intubated on mechanical ventilation, non-invasive positive pressure ventilation or days on nasal cannula by PH status. Respiratory status on the day of the echocardiogram was also evaluated (Table 3). Respiratory support was similar regardless of PH status on that day. There were also no differences by PH status for other measures of respiratory status that were included in the PROP study: respiratory severity index, room air (RA) challenge at 36 weeks gestational age, or respiratory morbidity at one year (Table 4).

In comparison to those infants who did not have an echocardiogram, infants who had an echocardiogram at 36 weeks were more likely have failed the RA challenge and spent more time on supplemental oxygen delivered by NC in the NICU (Supplemental Table 1 & 2).

Biochemical Markers

Of the 60 participants with echocardiographic data, 78% had biomarker specimens collected at all 4 prespecified time points. Using ordinal logistic regression, there was no evidence that an increase in plasma isoprostanes or isofurans were associated with PH severity at any time point (no PH, $n=49$; mild PH, $n=7$; or moderate PH, $n=4$; Table 5). Each model used a multiplicative scale for isoprostane or isofuran exposure and are interpreted as the relative odds of more severe PH comparing subjects differing in exposure by a factor of two. Analyzed by odds ratios relative to doubling in respective levels, there were no statistical differences at any time point for either plasma isoprostanes or isofuran levels by PH status.

Using a similar approach, the estimated odds ratios for a doubling of levels of the selected components of the nitric oxide pathway (urine NO_x, plasma arginine, plasma citrulline, and the arginine: ornithine + citrulline ratio) were analyzed (Table 6). Urine NO_x levels were corrected for urine Cr. The median urine Cr values for each of the four time points

(enrollment, day 7, 14, and 28) were similar: 0.09, 0.12, 0.1, and 0.12, respectively. For NOx, at time of enrollment (day of life 3) higher levels of NOx were associated with an increased odds of more severe PH (OR=3.62 for a doubling of NOx, 95% CI: [1.22, 10.7]). No associations of NOx with PH severity were observed at any of the subsequent time points. We also found no evidence of an association of arginine or the arginine to ornithine + citrulline ratio at any time point with PH severity. There was a significant, inverse association between citrulline measured at day 7 and PH severity (OR=0.29, 95% CI: [0.09, 0.86]) where higher levels of citrulline were found to be protective. That is, comparing two subjects who differ in their citrulline level at day 7 by a factor of 2, the subject with a higher citrulline levels is 3.44 times less likely to develop more severe PH (having a higher outcome category of PH). None of these infants with PH were receiving inhaled nitric oxide therapy at the time of these observations.

Within-subject changes in NOx and citrulline levels over time in subjects with PH (n=11) and without PH (n=49) are shown in Supplement Figure 2.

Discussion

Prior reports have estimated the prevalence of PH at 5–25% in the ELGAN population (7, 8, 14). Consistent with these reports, echocardiograms from ELGANs in our deeply phenotyped IMPROV cohort revealed that 18% of subjects had echocardiograms with features of PH; however, we did not find an association between PH and more severe clinical features, including markers of respiratory status. Prior studies reported a high incidence of PH in ELGANs with respiratory morbidities, such as those infants with a diagnosis of BPD (15, 16). Recent guidelines recommend that all premature infants with a diagnosis of BPD have an echocardiogram at 36 weeks corrected age to evaluate for the presence of PH (4). This recommendation comes from the previously referenced observations (12, 13) that a diagnosis of BPD is associated with changes in lung development, specifically decreased alveolarization and decreased pulmonary vascular growth, which is associated with an increased risk of PH in this premature infant population. However, our cohort shows that PH can develop in premature infants with a less severe respiratory phenotype. In careful interpretation of this data, we did not take this observation to contradict prior studies that have suggested a higher risk of PH in premature infants with significant lung disease, but rather that PH can also occur in premature infants with less severe respiratory morbidity. Further research is needed to identify all infants at risk for the development of PH.

The identification of PH in the premature infant population is difficult. Cardiac catheterization is the gold standard for diagnosis of PH, but in the vulnerable premature infant population this invasive procedure is less frequently completed for diagnostic purposes (17, 18). In our cohort, none of 11 infants with echocardiogram features of PH underwent cardiac catheterization. While it has been reported that the agreement between echocardiogram findings and cardiac catheterization in the premature infant population regarding PH severity can be suboptimal (19), it is also known that echocardiograms can provide multiple different parameters that can be used to indirectly estimate pulmonary pressures and there are ongoing attempts to better standardize these parameters (20). In our cohort, the echocardiogram parameter that was used most often to estimate pulmonary

pressures was interventricular septal wall flattening. While this parameter may not be as accurate in estimating PH severity, it has been shown to be reliable in predicting the presence of PH in this premature infant population (19). We also utilized LVSCI which is an echocardiographic measure to estimate the ratio of right ventricular pressure (RVP) to left ventricular pressure (LVP). It is calculated by the formula

$LVSCI = 4\pi (LV \text{ area}) \times \frac{100}{LV \text{ perimeter}}$ and by using regression analysis transseptal pressure ratio can be calculated $\frac{RVP}{LVP} = e^{(2.6 - 0.04 LVSCI)}$ (21). Overall, our echocardiogram data highlights the need for continued efforts to better standardize and validate echocardiogram analysis in the premature infant population.

All 11 of the infants with features of PH in this cohort were managed with supportive care and without PH specific medications, such as inhaled NO or sildenafil. Before 12 months chronological age, all of these 11 infants demonstrated a normal echocardiogram without features of PH. These observations reinforce the observation that for many of these infants, the natural history of PH is resolution in the first year of life (22). However, resolution in the first year of life is certainly not the case for all premature infants with PH (7, 8) and considering the observational nature of this study, it is unclear if the echocardiogram findings of these 11 infants with PH influenced clinical practice that may have led to resolution of PH by 12 months of age. Consequently, continued efforts are needed to better early identify premature infants with PH so that best clinical practice can be determined.

Validated biomarkers are heavily relied upon for clinical diagnosis and management in pediatrics because of the difficulty in obtaining more invasive measurements in such a vulnerable population (23, 24). The premature infant population is an especially vulnerable population within pediatrics and consequently requires careful consideration in this regard. In a complementary biomarker analysis, we found an association between PH and early elevation of urinary nitric oxide metabolites as well as decreased citrulline levels at day 7. Increased urinary NOx appeared to have the greatest association with PH severity and this was observed at time of enrollment (median day of life 3), suggesting the synthesis of NO was increased in the time period immediately after birth in infants who later developed PH. NO is made from arginine by one of three isoforms of nitric oxide synthase (NOS): endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS), which are expressed not only in the lung but in other tissues and organs. The source of NO and its metabolites measured in the urine cannot be determined by our study. A dose-dependent relationship between administration of inhaled nitric oxide and urinary NO metabolites has been shown previously (25); thus, the lung is one, but not the only, possible source of urinary NOx. NO is a growth factor important for normal lung development, but can also have deleterious effects, depending on concentration. Physiological doses are beneficial, maintaining blood flow in vessels and protecting against invading organisms. In high doses, NO can cause hypotension, mediate inflammation in sepsis and form cytotoxic peroxynitrite, particularly in the presence of high oxygen concentrations (26).

One source of the substrate, arginine, for NO production from eNOS (and most likely other isoforms of NOS) comes from the recycling of citrulline back to arginine through argininosuccinate synthase and argininosuccinate lyase (27, 28), as has been shown to occur

in neonatal piglet pulmonary artery endothelial cells and the lungs of young mice (29). In agreement with this observation, we observed decreased citrulline levels with worsening PH status at day of life 7. Further research is needed to validate these observations.

It has previously been reported that early pulmonary vascular disease, as assessed by echocardiogram in the first weeks of life, is associated with the development of BPD and late PH in preterm infants (30). Specifically, Mourani and colleagues found that the identification of PH on echocardiogram at day of life 7 was a risk factor for later development of BPD and PH at 36 weeks corrected gestational age. The current study was not designed to test that hypothesis. Future efforts may benefit from attempts to evaluate early neonatal echocardiograms along with biomarkers of the nitric oxide pathway and then associate these observations with long-term respiratory morbidity as well as PH.

A limitation of this study is that the echocardiograms were not obtained prospectively as part of the parent observational study. Rather, the echocardiograms used in this study were ordered by the clinician for various reasons, which were retrospectively analyzed in a blinded manner. We realize that an additional limitation is selection bias in that only 60 of 172 infants had an echocardiogram at 36 weeks corrected GA and it is possible that if this selection bias was not present we may have seen different associations between PH and respiratory morbidity. To address this possible bias we performed a supplementary analysis. Overall, the infants who had an echocardiogram at 36 weeks corrected GA were similar to those infants who did not, with the exceptions being that infants who had an echocardiogram spent more days on supplemental oxygen via nasal cannula and these infants were also more likely to fail the RA challenge (supplement table 2). It is possible that the presence of hypoxemia prompted the clinician to consider PH evaluation with an echocardiogram.

It is conceivable that infants without available echocardiograms had subclinical PH, or went on to develop PH. While the incidence of PH in our cohort was similar to other studies, the overall small number of infants with PH in this cohort (n=11), could have resulted in a failure to identify differences in respiratory morbidity between the two groups of our study. In addition, none of the infants had a cardiac catheterization to validate the echocardiogram findings, though this is not uncommon in the premature infant population. Future studies investigating clinical features and biomarkers for PH in the ELGANs population should utilize prospective echocardiograms obtained in a manner that allows for a focus on parameters that can be used for PH estimation. Despite these limitations, as previously discussed, our overall prevalence of PH was similar to what has been reported in the literature for this population.

In summary, in the Vanderbilt cohort (IMPROV) of the PROP study of premature infants, we observed an incidence of PH similar to what has previously been reported, but did not observe an association of PH with increased respiratory morbidity as has previously been reported. We observed an association of PH with increased levels of nitric oxide metabolites at time of enrollment and decreased levels of citrulline at day 7 of life. With further research, these NO pathway biomarkers obtained in the first week of life may be helpful for predicting later PH in this vulnerable population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Echocardiographic Features of Participants with Pulmonary Hypertension at 36 weeks Gestational Age

Participant	1	2	3	4	5	6	7	8	9	10	11
RA dilation	Mild	Mild	None	Mild	Mild	None	None	None	None	Mild	Mild
RV dilation	Mild	None	None	Mild	Mild	None	None	None	None	Mild	Mild
PA dilation	None	None	None	Mild	None	None	None	None	None	None	None
IVS flattening	Mild	Mild	Mild	Mild	Mod.	Mod.	Mild	None	Mild	Mod.	Mod.
RV wall motion	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
TR Present	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
Envelope	Incomp.	Incomp.	Incomp.	Incomp.	Incomp.	-	Incomp.	Incomp.	Incomp.	Incomp.	Incomp.
Gradient/SBP	-	-	22.9/78	34/83	26/74	-	37/69	18/84	11.5/84	14.6/90	35/92
Est. RVP	-	-	28	39	31	-	42	23	16.5	19.6	40
RVP/LVP	-	-	0.35	0.46	0.41	-	0.6	0.27	0.19	0.21	0.43
PDA present	No	No	No	No	Yes	No	No	Yes	No	Yes	No
Direction					L->R			L->R			
Gradient/SBP								41/84		27/90	
LVSCI	91.6	87.6	95.7	93	85.5	89.2	87.6	96.4	91.5	89.2	88.3
PH Severity	Mild	Mild	Mild	Mild	Mod.	Mod.	Mild	Mild	Mild	Mod.	Mod.

Clinical echocardiograms (n=60) obtained at 36 weeks corrected gestational age (+/- 1 week) were blindly analyzed by a single pediatric cardiologist for features of pulmonary hypertension. Ten infants were found to have a least one feature of pulmonary hypertension as described in the table. Mod., moderate; Incomp., incomplete; TR, tricuspid regurgitant; LVSCI, left ventricular systolic circular index.

Table 2

Participant Demographics

Covariate	PH Present (n=11)	PH Not Present (n=49)	p-value
Gestational age at birth (weeks)	27 [25, 28]	26 [25, 27]	0.56
Gender (female)	4 (36%)	18 (37%)	0.98
Birth weight (grams)	980 [792, 1170]	890 [710, 1060]	0.33
Weight gain per day (grams/day)	21 [18, 25]	22 [20, 23]	0.80
Length of NICU Hospitalization (days)	70 [67, 92]	89 [70, 120]	0.23
Days Intubated	1 [1, 8.5]	8 [1, 25]	0.12
Days of HFNC	39 [33, 50]	29 [17, 45]	0.19
Days on CPAP	0.5 [0, 3.5]	2.0 [0, 8]	0.31
Days on low flow NC	15 [8, 26]	19 [6, 36]	0.41

Participant clinical demographics by pulmonary hypertension status. Continuous variables are expressed as median with IQR. Statistical testing by Wilcoxon signed rank test, statistical significance (*) at $p < 0.05$.

NICU, neonatal intensive care unit; HFNC, high-flow nasal cannula (Vapotherm© used in this cohort); CPAP, non-invasive continuous positive airway pressure; NC, nasal cannula, generally defined as less than 2L.

Table 3

Respiratory Support on Day of Echocardiogram at 36 weeks Gestational Age

Respiratory Support	PH Present (n=11)	PH Not Present (n=49)
Room-air (no support)	4/11 (36%)	19/49 (39%)
Nasal Cannula		
< 0.25 LPM	3/11 (27%)	9/49 (18%)
0.25 LPM – 0.75 LPM	0 (0%)	2/49 (4%)
> 0.75 LPM	3/11 (27%)	9/49 (18%)
HFNC	1/11 (9%)	8/49 (16%)
Intubated/mechanical ventilation	0 (0%)	2/49 (4%)

Respiratory support on day of echocardiogram completed at 36 weeks gestational age by pulmonary hypertension status. NC, nasal cannula; HFNC, high flow nasal cannula (Vapotherm© used in this cohort)

Table 4

PROP Respiratory Outcomes by PH Status

	PH Present (n=11)	PH Not Present (n=49)
RA Challenge at 36 weeks GA (failed = BPD)	7/11 (64%)	27/49 (55%)
PRD at 1 year (n=47)	8/10 (80%)	27/37 (73%)

Room-air (RA) challenge and postprematurity respiratory disease (PRD) at 1 year are both dichotomous variables. PROP, premature respiratory outcomes program; PH, pulmonary hypertension; GA, gestational age.

Table 5

Associations of Plasma Isoprostanes and Plasma Isofurans with PH Severity at 36 Weeks Corrected Gestational Age

Time point	Marker	N	Odds ratio for worsening PH status relative to doubling of biomarker level	p-value
Birth	Isoprostane	47	0.69 [0.39, 1.23]	0.21
	Isofuran	47	0.95 [0.58, 1.53]	0.82
Day 7	Isoprostane	60	0.97 [0.59, 1.58]	0.89
	Isofuran	60	0.84 [0.52, 1.36]	0.49
Day 14	Isoprostane	57	1.26 [0.64, 2.50]	0.51
	Isofuran	57	0.89 [0.56, 1.42]	0.63
Day 28	Isoprostane	53	0.71 [0.34, 1.47]	0.36
	Isofuran	53	0.89 [0.63, 1.25]	0.49

Isoprostane and Isofuran levels are expressed as odds ratio, with lower and upper confidence intervals. Odds are relative to increased severity of PH with doubling of the biomarker level. Statistical significance (*) at $p < 0.05$.

Table 6

Associations of NOx, Arginine, and Citrulline Levels with PH Severity at 36 Weeks Corrected Gestational Age

Time point	Marker	N	Odds ratio for worsening PH status relative to doubling of biomarker level	p-value
Birth to day 3	NOx	47	3.62 [1.22, 10.74]	0.02*
	Arginine	46	0.82 [0.38, 1.80]	0.63
	Citrulline	46	0.91 [0.34, 2.41]	0.85
	Arg:OrnCit	46	0.58 [0.19, 1.78]	0.34
Day 7	NOx	60	1.67 [0.74, 3.77]	0.22
	Arginine	60	1.01 [0.54, 1.89]	0.98
	Citrulline	60	0.29 [0.09, 0.86]	0.03*
	Arg:OrnCit	60	1.00 [0.50, 1.97]	0.99
Day 14	NOx	58	1.38 [0.84, 2.27]	0.21
	Arginine	58	0.72 [0.42, 1.24]	0.24
	Citrulline	58	0.94 [0.42, 2.09]	0.88
	Arg:OrnCit	58	0.69 [0.26, 1.87]	0.47
Day 28	NOx	58	1.23 [0.84, 1.81]	0.29
	Arginine	52	0.50 [0.22, 1.13]	0.10
	Citrulline	52	2.22 [0.71, 6.91]	0.17
	Arg:OrnCit	52	0.49 [0.19, 1.26]	0.14

Levels are expressed as odds ratio, with lower and upper confidence intervals. Odds are relative to increased severity of PH with doubling of the biomarker level. Statistical significance (*) at $p < 0.05$.