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Plasma Lipid Metabolites are Associated with Gestational Age but Not Bronchopulmonary Dysplasia

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Abstract

Aim—To test the hypothesis that plasma lipid metabolite levels in premature infants are associated with the development of bronchopulmonary dysplasia (BPD). The studies also tested a secondary hypothesis that plasma lipid metabolite levels were correlated with gestational age.

Methods—Infants born less than 32 weeks gestation were enrolled during the first 72 hours of life. Plasma samples were obtained and lipid levels were measured by LC-MS/MS. Clinical data were collected to determine infant outcomes and BPD diagnosis.

Results—Following adjustment for confounders, lipid levels were not associated with BPD; however, levels of specific lipid metabolites were correlated with gestational age.

Conclusion—Immature lipid metabolism pathways in premature infants may contribute to the pathogenesis of BPD and other diseases.

Keywords

bronchopulmonary dysplasia (BPD); lipids; gestational age; LC-MS/MS

Introduction

Premature birth is the leading cause of infant mortality and morbidity in the United States representing 12.3% of all live births in the United States in 2008 (1). The lungs of premature infants are structurally and biochemically immature and are often exposed to antenatal inflammation and/or postnatal oxygen with or without mechanical ventilation. Inflammation-mediated lung injury contributes to the development of bronchopulmonary dyplasia (BPD) (2, 3). For the purposes of this study, BPD is defined as requiring respiratory support at 36 weeks corrected gestational age and is characterized by arrested lung development with fewer and larger alveoli (2). Infants with BPD often display persistently abnormal pulmonary function and other medical problems that impair their overall health (4–7).

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Inflammatory responses, such as those observed in infants that develop BPD, can initiate a premature release of fatty acids from membrane phospholipids making them available to be further metabolized into biologically active products (8). Cleaved fatty acids can be metabolized by 1) cyclooxygenases to form prostaglandins (PG) or thromboxane (TBX), 2) lipoxygenases form eicosatetraenoic acids (HETEs), and 3) cytochrome P450-mediated processes form epoxyeicosatetraenoic acids (EETs). More specifically, 5-lipoxygenase forms a unique class of mediators called leukotrienes (LT). Alternatively, metabolites such as the HETEs or 8-isoPGF2α can be formed by free radical oxidation. Furthermore, specific lipid metabolites are elevated in infants diagnosed with hyperoxic lung injury and/or BPD (9–11).

Previous studies have analyzed tracheal aspirates to identify molecules that are associated with or contribute to the development of BPD (12–14). However, as supportive care for preterm infants has progressively moved away from prolonged endotracheal intubation and mechanical ventilation, the acquisition of tracheal aspirate samples is frequently impossible in all but the most severely ill infants. Although plasma may not directly reflect lung physiology, it is a readily available sample that may indirectly reflect inflammatory changes in the lung.

The present studies tested the overall hypothesis that plasma lipid metabolite profiles of premature infants correlate with the development of BPD. Our preliminary findings led us to test a second hypothesis that the levels of plasma lipid metabolites in premature infants correlate with gestational age.

Study Design

Patient Enrollment

The studies described in this proposal were approved by the Institutional Review Board of The Research Institute at Nationwide Children's Hospital. Patients were enrolled by written informed consent of the parents from neonatal intensive care units at Nationwide Children's Hospital and The Ohio State University Hospital, Columbus, Ohio between May 2005 and December 2008. Eligible infants included those born <32 weeks gestation and enrolled within the first 72 hours of life. Premature infants that did not develop BPD served as the control group. Infants with congenital defects that would alter the standard course of care for prematurity were excluded (i.e. congenital heart disease, facial or GI malformations, genetic abnormalities).

Two hundred eighty-three pre-term infants were enrolled. Of these, eight did not survive long enough to be diagnosed with BPD and 3 did not meet the gestational age criteria (gestational age was corrected after enrollment) leaving a total of 272 infants for this study.

Sample and data collection

Approximately 0.5 mL of blood was drawnbetween 0 and 72 hours of life depending upon the time of enrollment. All samples were placed at 4° C immediately after collection, the cells were separated from the plasma and the plasma was stored at −80° C.

Laboratory Analyses

Lipid metabolites were measured on plasma samples by liquid chromatography/mass spectrometry (LC-MS/MS) techniques using Multiple Reaction Monitoring (MRM) and stable isotope dilution for quantification. The ions monitored for quantification are listed in Supplemental Table 1. Specifically, 100 μL of plasma was spiked with a deuterated eicosanoid internal standard solution then extracted with organic solvents ($CHCl_3$:MeOH).

Samples were injected into an Applied Biosystems 4000 QTrap LC/MS/MS equipped with a Shimadzu HPLC. After HPLC separation, the samples were analyzed by mass spectra in negative ion mode. Individual calibration curves were generated for each class of molecules and sample concentrations were calculated using isotope dilution corrections.

Statistical Analyses

For statistical analyses the infants were divided using the following criteria: control (*not requiring respiratory support at 36 weeks corrected gestational age*) or BPD (*requiring respiratory support at 36 weeks corrected gestational age*). Respiratory support was defined as any breathing assistance including supplemental oxygen or assistance devices, mechanical ventilation, nasal CPAP, or cannula. As a screening technique, a total of 36 lipids were initially measured; however, many of the metabolites were below the lower limit of detection due to limited sample volume. In our final analyses, 16 lipid metabolites met the inclusion criteria of having values above the lower limits of detection for at least half of the samples (limits of detection were defined as >0.005 pmol/mL plasma). Four combinations of metabolites (precursor and products) were also considered. In total, 20 lipid products were analyzed statistically (Supplemental Figure 1).

For the first hypothesis, logistic regression was used to model odds of BPD as a function of lipid levels and GEE was used to account for the clustering of multiple infants to a single mother, e.g., twins, triplets (15). Twenty different models were built corresponding to the twenty different lipid products using a forward selection process. Multiple metabolites were not included simultaneously in any model because their interdependence raised concerns about colinearity and interpretability of associations. To account for confounding, independent variables related to BPD status ($p < 0.2$) were added in a stepwise fashion in order of their effect on the adjusted odds ratios relating metabolite levels to BPD. However, due to sample size limitations, only the two most important confounders were added (Table 1). Four of the continuous variables identified as potential confounders (gestational age, birth weight, birth head circumference, and birth length) were highly correlated; consequently, only gestational age was considered. We used the Benjamini-Hochberg method (16) to control false discovery rate across our twenty models resulting in the generation of q-values which were compared to a 0.05 significance level.

For the second hypothesis, analyses were performed to explore the relationship between lipid levels and gestational age. Twenty different GEE linear regression models were used to examine the relationships between metabolite level (dependent variable) and gestational age (independent variable) and the Benjamini-Hochberg method was applied as previously described. All analyses were conducted using SAS version 9.1 (SAS Inc., Cary, NC).

Results

Table 1 summarizes the characteristics of the infants in our study, stratified by BPD status. Of the 272 infants in the present study, 88 infants developed BPD, and 184 infants did not. The first step forward selection process indicated that adjustment for gestational age had the greatest effect on the estimated association between plasma lipid levels and BPD diagnosis while adjustment for gender had the second greatest effect. Therefore, both variables were included in our final analyses. While ibuprofen/indomethacin exposure and IUGR were identified as a potential confounders, only twelve infants (9 BPD, 3 controls) received ibuprofen/indomethacin prior to the blood draw used in our analyses and only 29 were classified IUGR (7 BPD, 22 controls, using the CDC growth chart, 2000) leaving us with an insufficient sample size to include these variables in our analyses.

Odds ratios relating plasma lipid levels (pmol/mL) and BPD diagnosis are provided in Table 2. Prior to adjustment for gestational age and gender, ten of the measured plasma lipid metabolites were significantly related to a diagnosis of BPD. However, once adjusted for gestational age and gender, the q-values for these metabolites were no longer significant. The change in the q-values following adjustments led us to speculate that expressions of these specific lipid metabolites are developmentally regulated. Thus, we tested a second hypothesis that plasma lipid levels are correlated with gestational age.

As indicated in Table 3, expressions of nine of the lipid metabolites measured were correlated with gestational age. When combined, TXB_2 and its products 11-dehydro TXB_2 and 2,3-dinor TXB_2 were correlated with gestational age as were the combination of LTB_4 and 20-hydroxy LTB_4. Data indicating range, mean values of each metabolite, and number of infants at each gestational age are presented in Supplemental Table 2.

Discussion

Our studies investigated the relationship between formation of plasma lipid metabolite levels in premature infants and the development of BPD. While we initially identified associations, these were no longer significant following correction for gestational age and gender in our analyses. Plasma PGE_2 and $TBXB_2$ levels have previously been measured in infants at days of life 1, 3, and 7 (17); however, similar to the present study, no differences were detected. Though the authors did not control for gestational age, they speculated that maturational changes in lipid metabolism might account for their findings and our data support their speculation.

The secondary analyses indicated that several individual or combined lipid metabolites were correlated with gestational age at birth. It is important to note that eight of the eleven identified metabolites associated with gestational age can be formed through activity of lipoxygenase enzymes. One interpretation of these data is that extremely preterm infants may be deficient lipoxygenase pathways enzymes.

HETEs can be generated by multiple mechanisms including free radical oxidation or oxidation through the activities of individual lipoxygenases (18). Our data indicates that absolute quantities of HETEs were higher in the infants that developed BPD and most were correlated with gestational age. HETEs are biologically active molecules that increase in response to injury and repair and modulate cell proliferation (19). The increases in these products as a function of gestational age suggest that these pathways are adaptive rather than injurious. LTB_4 is the most potent chemotactic agent produced from arachidonic acid (20), is often associated with neutrophil adhesion and infiltration at sites of inflammation, and likely contributes significantly to hyperoxic lung injury (21). Increased endothelial cell permeability is a significant event associated with hyperoxic lung injury (22). EETs are formed by cytochrome P450 activity and play a direct role in regulating endothelial cell permeability (23), vasodilatation, and Ca^{2+} entry into the lung microvasculature.

The association between gestational age and expressions of these lipid metabolites suggests that the pathways responsible for their production are regulated through processes during intrauterine growth. The developmental regulation of lipid metabolism could be especially significant in the extremely preterm infant that is more likely to develop morbidities such as BPD. Deficiencies in biologically active lipids in extremely preterm infants may contribute to inappropriate responses to inflammation, inflammatory stimuli, or tissue repair, resulting in further or permanent tissue injury.

The prostaglandin product 8-iso-$PGF_{2\alpha}$, a product of free radical oxidation, has previously been measured in infants at risk for the development of BPD. Cord blood plasma levels of 8-

iso-$PGF_{2\alpha}$ were higher in preterm infants than in term infants and directly correlated with poor outcomes (24). Studies by Ahola et al. (25) indicated that plasma 8-iso-$PGF_{2\alpha}$ levels at day of life 3 and 7 were highest in the extremely low birth weight infants that developed BPD and correlated with oxygen support at 7 and 14 days. Unlike the present study, however, Ahola et al. did not correct for gestational age. Following correction for gestational age, our data indicated no correlation between 8-iso-$PGF_{2\alpha}$ and BPD development but did indicate a positive correlation with gestational age. While our unadjusted results are in agreement with Ahola et al., the loss of significance following adjustment for confounders highlights the importance of appropriately powered studies that enable such adjustments.

The strengths of this study include the prospective study design, the relatively large sample size, the statistical analyses, and the specificity of the analyses enabled by the use of mass spectrometry. Limitations worth noting include the lack of sensitivity to detect some plasma lipid metabolites as evidenced by the number of candidate biomarkers that were below the limits of detection. Secondly, while 0–72 hours were chosen for sample collection, later time points may have been more informative and may have better correlated with disease outcomes. The present studies involved a greater number of patients than in previously published studies allowing us to control for multiple confounding variables in the statistical analyses; however, the use of even more sophisticated statistical modeling was still limited by the cohort size.

Though we were unable to identify correlations between plasma lipid metabolites and the development of BPD, we did identify significant associations with gestational age indicating that pathways governing lipid metabolism are likely to be developmentally regulated in prematurely born infants. These data represent the first attempt to assess lipid metabolites in a relatively large cohort of preterm infants which incorporated the use of statistical analyses adjusting for confounders. While our unadjusted analyses might have led us to conclude that plasma lipid products are associated with the development of BPD, the prospective nature of our study and our ability to account for patient characteristics led to an entirely different and admittedly less exciting conclusion. Nonetheless, we speculate that plasma lipid mediators likely contribute to altered responses in extremely premature infants and highlight the need for focused studies to identify the events that underlie developmental maturation of lipid metabolic pathways and their contribution toward the development of BPD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BPD	bronchopulmonary dysplasia
AA	arachidonic acid
GEE	generalized estimating equations
ALD	above limits of detection

PG	prostaglandin
LT	leukotriene
TBX	thromboxane
HETE	hydroxyeicosatetranoic acid
EET	epoxyeicosatetranoic acid

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Key Notes

A prospective study evaluated the association between plasma lipid metabolites and BPD. Although correlations were found between lipid metabolites and BPD in unadjusted analyses, statistical adjustments for gestational age and gender resulted in no correlations. Eleven lipid metabolites were correlated with gestational age, indicating a developmental maturation in lipid metabolism. We speculate that plasma lipid mediators indicate altered responses in extremely premature infants that could contribute to later morbidities.

Table 1

Summary of Population Characteristics.

Characteristic		**Total n=272**	**No BPD n=184**	**BPD n=88**	**p-value#**
Mean (std dev) Gestational Age in weeks		28.5 (2.4)	29.6 (1.8)	26.2 (2.0)	**<0.001**
Mean (std dev) Maternal Age in years		27 (6)	27 (6)	26 (6)	0.077
Infant Gender (n (%))	Male	148 (54.4)	94	54	0.112
	Female	124 (45.6)	90	34	
Maternal Race (n (%))	Non-Caucasian	55 (20.2)	40	15	0.373
	Caucasian	217 (79.8)	144	73	
Chorioamnionitis * (n (%))	Yes	88 (32.4)	59	29	0.885
	No	161 (59.2)	109	52	
	Unknown	23 (8.5)	16	7	
Maternal Steroids (n (%))	Yes	240 (88.2)	170	70	**0.002**
	No	32 (11.8)	14	18	
Multiple Birth (n (%))	Yes	67 (24.6)	48	19	0.458
	No	205 (75.4)	136	69	
Prenatal Care * (n (%))	Yes	256 (94.1)	175	81	0.341
	No	13 (4.8)	8	5	
	Unknown	3 (1.1)	1	2	
Para ** (n (%))	1	99 (36.4)	58	41	**0.019**
	2	94 (34.6)	66	28	
	3	51 (18.8)	37	14	
	4+	28 (10.3)	23	5	
Gravida ** (n (%))	1	87 (32.0)	56	31	0.445
	2	77 (28.3)	59	18	
	3	49 (18.0)	28	21	
	4+	59 (21.7)	41	18	

p-values calculated using GEE logistic regression.

* 'No' and 'Unknown' were combined for comparison purposes.

** Para 2, 3 and 4+ were combined for comparison purposes.

IUGR was considered but the sample size was insufficient for statistical analysis (total 29, BPD 9)

Table 2

Unadjusted and Adjusted Estimates of the Effects of Measured Lipid Metabolite Levels on BPD Status.

		No BPD		BPD		--Unadjusted--		--Adjusted--	
	Analyte	**Mean**	**SE**	**Mean**	**SE**	**Odds Ratio**	**q-value**	**Odds Ratio**	**q-value**
1	LTB_4	0.22	0.040	0.81	0.125	0.229	**0.004**	0.509	0.446
2	PGF_{2a}	35.24	0.681	35.44	0.578	0.996	0.847	1.016	0.844
3	TXB_2	4.68	1.342	29.68	4.077	0.966	**0.031**	0.980	0.276
4	5-S-Hete	7.06	0.484	8.94	0.495	0.944	**0.032**	0.992	0.847
5	8-S-Hete	2.58	0.176	3.75	0.200	0.767	**0.003**	0.874	0.446
6	9-S-Hete	13.82	1.593	21.11	1.586	0.979	**0.010**	0.990	0.748
7	8,9-Eet	0.62	0.119	1.08	0.117	0.797	0.229	0.885	0.847
8	9-S-Hode	237.06	7.561	231.46	7.666	1.001	0.801	1.001	0.844
9	11-S-Hete	6.69	0.677	9.98	0.778	0.947	**0.010**	0.977	0.754
10	11, 12-Eet	0.57	0.080	0.81	0.088	0.732	0.136	0.771	0.748
11	12-S-Hete	34.82	4.727	90.01	11.562	0.991	**0.021**	0.996	0.276
12	13-S-Hode	69.62	7.352	69.56	8.835	1.000	0.959	1.000	0.847
13	15-S-Hete	3.36	0.264	6.34	0.432	0.834	**<0.001**	0.922	0.276
14	5, 6-Eet	3.35	0.155	3.53	0.125	0.927	0.525	1.040	0.847
15	14, 15-Eet	1.32	0.127	1.29	0.082	1.039	0.847	0.919	0.847
16	8-iso-$PGF_{2\alpha}$	11.64	1.648	10.79	1.056	0.974	0.337	0.981	0.844
17	LTB_4 + 20-hydroxy LTB_4	0.21	0.038	0.80	0.121	0.259	**0.003**	0.533	0.446
18	PGF_{2a} + 11b-$PGF_{2\alpha}$ + 13,14-dihydro-15 keto $PGF_{2\alpha}$	33.54	1.217	36.59	0.941	0.977	0.136	1.000	>0.999
19	TXB_2 + 11-dehydro TXB_2 + 2,3-dinor TXB_2	5.33	1.245	30.04	3.968	0.967	**0.023**	0.982	0.276
20	8-iso-$PGF_{2\alpha}$ + 2, 3-dinor-8-iso-$PGF_{2\alpha}$	4.92	0.881	6.06	0.676	1.009	0.801	1.008	0.847

Data were adjusted for gestational age and gender.

Table 3

Regression Analyses for Continuous Metabolite Levels Modeled on Gestational Age.

	Analyte	Slope	SE	q-value
1	LTB_4	0.022	0.008	**0.019**
2	$PGF_{2\alpha}$	0.037	0.025	0.166
3	TXB_2	−0.836	0.894	0.411
4	5-Hete	0.075	0.020	**0.001**
5	8-Hete	0.037	0.008	**<0.001**
6	9-Hete	0.224	0.064	**0.001**
7	8,9-Eet	0.013	0.005	**0.026**
8	9-Hode	−0.002	0.247	0.995
9	11-Hete	0.104	0.033	**0.004**
10	11,12-Eet	0.009	0.004	0.056
11	12-Hete	1.525	0.371	**<0.001**
12	13-Hode	0.068	0.244	0.868
13	15-Hete	0.083	0.018	**<0.001**
14	5,6-Eet	0.010	0.006	0.155
15	14,15-Eet	−0.000	0.004	0.995
16	8-iso-$PGF_{2\alpha}$	0.318	0.003	**<0.001**
17	LTB4 + 20-hydroxy LTB4	0.017	0.005	**0.003**
18	$PGF_{2\alpha}$ + 11β-$PGF_{2\alpha}$ + 13,14-dihydro-15-keto-$PGF_{2\alpha}$	0.051	0.032	0.155
19	TXB_2 + 11-dehydro TXB_2 + 2,3-dinor TXB_2	0.736	0.152	**<0.001**
20	8-iso-$PGF_{2\alpha}$ + 2, 3-dinor-8-iso-$PGF_{2\alpha}$	0.048	0.032	0.166