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Peptide Growth Factors in Tracheal Aspirates of Mechanically Ventilated Preterm Neonates

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ABSTRACT

Basic fibroblast growth factor (bFGF or FGF-2), vascular endothelial growth factor (VEGF), and endothelin-1 (ET-1) are peptide growth factors (PGF) mediating normal lung development, maturation, injury, and repair. These PGF may therefore be involved in the pathogenesis of bronchopulmonary dysplasia (BPD). We hypothesized that elevated levels of these PGF in tracheal aspirates would be associated with a) BPD and/or death; b) markers of cell injury and apoptosis; and c) chorioamnionitis, a risk factor for BPD. Tracheal aspirates collected in 29 preterm (<34 wk gestation, 500–2000 g birth weight), mechanically ventilated infants on d 1 of life were assayed for PGF and histone-associated DNA fragments by ELISA and for LDH by enzyme assay. Clinical and pathologic examination was performed for chorioamnionitis. BPD was defined as oxygen requirement/mechanical ventilation at 28 d postnatal age. The birth weight (mean ± SE) was 1009 ± 85 g and median gestational age was 26 wk (range, 22–33). Eighteen infants died or developed BPD. bFGF levels were elevated in infants who died or developed BPD [median (25%,75%) level of 36 (23, 44) pg/mL *versus* 14 (6, 30) in the survivors without BPD, $p = 0.01$]. bFGF levels correlated with apoptosis ($r = 0.73$, $p < 0.001$) and LDH levels ($r = 0.59$, $p < 0.001$). VEGF and ET-1 levels were not associated with apoptosis or with BPD/death. PGF levels were not associated with chorioamnionitis. We conclude that elevated bFGF levels in the preterm trachea correlate with BPD/death and markers of cell injury and apoptosis but not with chorioamnionitis. We speculate that bFGF may play a role in the development of BPD. **(*Pediatr Res* 53: 240–244, 2003)**

Abbreviations

bFGF, basic fibroblast growth factor (fibroblast growth factor-2)
BPD, bronchopulmonary dysplasia
ET-1, endothelin-1
LDH, lactate dehydrogenase
PGF, peptide growth factor
VEGF, vascular endothelial growth factor

Mortality and BPD are common in preterm neonates requiring mechanical ventilation. Factors involved in the development of BPD include impairment of normal lung development in association with lung injury and repair. PGF, including bFGF (FGF-2) (1,2), other members of the fibroblast growth factor family such as FGF-7/keratinocyte growth factor (KGF) and FGF-10 (3–7), epidermal growth factor (EGF) (8, 9), endothelin-1 (10, 11), platelet derived growth factor (PDGF) (6, 12, 13), transforming growth factor β-1 (7, 9), and VEGF (14) are important in lung anatomic and functional development. There is preliminary evidence that ET-1 (15, 16) and VEGF (14, 17) may be involved in the pathogenesis of BPD in infants, and that bFGF is elevated in the airway epithelial lining fluid in adults with acute respiratory distress syndrome (ARDS) (18). We performed a pilot study to test the hypotheses that elevated levels of bFGF, ET-1, and VEGF in tracheal aspirates of mechanically ventilated preterm neonates would be associated with a) BPD and/or death; b) markers of cell injury and apoptosis; and c) chorioamnionitis, a risk factor for BPD.

METHODS

Subjects. This was a prospective study on 30 preterm infants admitted to the Regional Neonatal Intensive Care Unit at the University of Alabama at Birmingham from April to September 2000. Infants were eligible if they were <34 wk gestational age and weighed 501–2000 g, and received mechanical ventilation on the first day of life. The protocol was approved by the institutional review board, and informed consent was obtained for each participant before entry into the study.

Definitions. A clinical diagnosis of chorioamnionitis was based on the presence of one or more of the following criteria: preterm prolonged rupture of membranes >24 h, maternal fever >38°C, maternal sepsis, foul-smelling or purulent amniotic fluid, and uterine tenderness. Pathologists masked to the

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study established a histologic diagnosis of maternal chorioamnionitis.

BPD was defined as the need for supplemental oxygen or mechanical ventilation at 28 d postnatal age, in association with radiologic changes consistent with BPD (BPD_{28d}). Additional analysis was also performed using the alternative definition of BPD as supplemental oxygen or mechanical ventilation at 36 wk postmenstrual age (BPD_{36wk}). Death was defined as mortality before hospital discharge or 120 d hospital stay.

Tracheal aspirates. Tracheal aspirates were collected after informed consent was obtained. Aspiration of the endotracheal tube was only undertaken when clinically indicated. All tracheal aspirates were collected before 24 h of age, after surfactant administration. A standardized procedure was used. After endotracheal instillation of 1 mL/kg of normal saline, three to five breaths were given with the ventilator or flow-inflating resuscitation bag. A premeasured suction catheter was advanced through the endotracheal tube and suctioning performed just distal to the tip of the tube. Fluid was aspirated into the Leuken trap, placed on ice, and processed within 20–40 min. Samples with visible blood were discarded. All samples were diluted with 3 mL of PBS and centrifuged at 12,000 × *g* for 10 min at 4°C. Supernatants were stored at −70°C until analyzed for PGF by ELISA. As recommended by the European Respiratory Task Force on Bronchoalveolar Lavage in children (19), we did not correct our results for the dilution during the lavage procedure, and expressed our data per milliliter of tracheal aspirate, as results obtained by urea dilution or correction for protein concentration may show more inaccuracy because of increased vascular permeability and lung inflammation in sicker infants. All assays were performed by investigators masked to outcome until completion of the assays. Wright-stained smears of cytospin slides of tracheal aspirates were examined for cell density (cells/high power field), percentage of the cells that were white blood cells (WBC), and differential WBC count (percentage polymorphonuclear leukocytes) by an observer masked to patient identity and clinical outcome. The average number of polymorphonuclear leukocytes (neutrophils) was then calculated from these indices.

Analysis for PGF. PGF were measured by ELISA. bFGF was estimated using a high-sensitivity quantitative sandwich enzyme immunoassay (ELISA) obtained from R & D Systems (Minneapolis, MN, U.S.A.). This assay used MAb specific for bFGF and had a sensitivity of 0.2 pg/mL, with no cross-reactivity or interference from acidic FGF, FGF-4, -5,-6, or -7. VEGF was also assayed by a similar quantitative sandwich enzyme immunoassay from R & D Systems. The sensitivity of the VEGF assay was 10 pg/mL, without significant cross-reactivity with or interference from other PGF. ET-1 was estimated using an ELISA obtained from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). This ELISA was specific for human ET-1 (and ET-2), with <0.1% cross-reactivity to Big ET-1 or ET-3. The sensitivity of this assay was 0.5 fmol/well (12.5 pg/mL). Intra-assay variability in all the PGF assays was <10%.

Quantitation of apoptosis and cell injury. Apoptosis was estimated by a quantitative sandwich enzyme immunoassay (Cell Death Detection ELISA, Roche Diagnostics, Indianapolis, IN, U.S.A.) using mouse MAb directed against DNA and histones, permitting the determination and quantitation of mono- and oligonucleosomes in the supernatant of the tracheal aspirate. The mono- and oligonucleosomes are evidence of DNA fragmentation produced during apoptosis, and are responsible for the "DNA-laddering" on agarose gels.

Cell injury was estimated by measurement of cytoplasmic LDH in the cell-free component (supernatant) of the tracheal aspirates, which is an indicator of membrane integrity. Damaged cells with loss of cell membrane integrity leak LDH into the surrounding milieu. LDH was measured by enzyme assay (Sigma Chemical, St. Louis, MO, U.S.A.).

All assays were performed using varying dilutions of positive controls. All PGF assays, the apoptosis assay, and the LDH assay have been previously validated in our laboratory on other samples.

Clinical care. All infants were treated in accordance with standard neonatal intensive care unit protocols. As many infants required mechanical ventilation for only a few days, sequential samples over a period of days or weeks could not be collected.

Statistical analysis. The *t* test or the Fisher exact test were used as indicated for the comparison of patient characteristics. The Mann-Whitney rank sum test was used for the comparison of PGF levels in infants with normal outcome *versus* those who died or developed BPD. The Pearson or the Spearman correlation coefficients (depending on the whether the distribution was normally distributed or not) were used to assess the relationship between PGF levels and apoptosis or LDH. The software package SigmaStat v.2.03 (Jandel Scientific, San Rafael, CA, U.S.A.) was used for statistical analysis. All data are shown as median (25%,75%) unless otherwise indicated.

RESULTS

Thirty premature infants were studied. Twenty-nine tracheal aspirates could be analyzed (one sample was lost during processing). The birth weight was 1009 ± 456 g (mean ± SD; range, 517–1910 g), and the median gestational age was 26 wk (mean ± SD, 26.6 ± 2.5 wk; range, 22–33 wk). Eighteen of 29 (62%) infants were male and 12 of 29 (41%) were Caucasian. Eighteen of 29 (60%) had histologic and clinical evidence of chorioamnionitis. Eighteen of 29 infants developed BPD_{28d}, and nine developed BPD_{36wk}. Of the seven infants who died, four died after developing BPD_{28d} but before developing BPD_{36wk}. Infants who developed BPD_{28d} or died were of a lower gestational age (median, 25.6 wk *versus* 28 wk; $p = 0.003$) and had a lower birth weight (mean ± SD, 812 ± 220 g *versus* 1239 ± 303 g; $p < 0.001$), compared with those with a normal outcome. Antenatal steroids were received by 20 infants (five of 11 with normal outcome, and 15 of 18 who developed BPD_{28d} or died; $p = 0.048$) and intrapartum antibiotics by 22 infants (six of 11 with normal outcome, and 16 of 18 who developed BPD_{28d} or died; $p = 0.07$).

bFGF. bFGF was significantly elevated in infants who died or developed BPD_{28d} (Table 1, Fig. 1). The median level of bFGF was 36 pg/mL in infants who died or developed BPD_{28d} *versus* 13.5 pg/mL in those who survived without BPD_{28d}. bFGF levels in the nine infants who died or developed

Table 1. *PGF levels in tracheal aspirates of mechanically ventilated preterm infants in relation to the outcome of BPD (O_2/mechanical ventilation at age 28 d) and/or death*

PGF	No BPD/death (n = 11)	BPD/death (n = 18)	p Value*
bFGF	13.5 (6, 30)	36 (23, 44)	.01
VEGF	290 (101, 608)	114 (11, 311)	.13
ET-1	57 (31, 135)	77 (60, 105)	.77

Values given in picograms per milliliter [median (25%, 75%)].
* Mann-Whitney rank sum test.

BPD_{36wk} [39 pg/mL (11, 52)] showed a trend to be elevated compared with survivors without BPD_{36wk} [22.5 pg/mL (9, 35); $p = 0.07$]. When only infants who died were considered, bFGF was elevated [42 pg/mL (19, 57)] compared with survivors [22.5 pg/mL (9, 36), $p < 0.05$]. bFGF was moderately inversely correlated with birth weight (Spearman's $r = -0.55$, $p = 0.002$) and gestational age (Spearman's $r = -0.45$, $p = 0.02$), indicating that infants with lower birth weight (who are at higher risk of mortality) had higher levels of bFGF. To identify whether the relationship with birth weight was the reason bFGF was associated with mortality, a backward stepwise regression was performed with mortality as the dependent variable and birth weight, gestational age, and bFGF levels as independent variables. bFGF was most strongly associated with mortality in this model, and birth weight and gestation did not add significantly to the predictive ability of bFGF. When only survivors were considered, higher bFGF levels showed a trend toward association with BPD_{28d}. bFGF level in survivors with BPD_{28d} was 30 pg/mL (22, 38) and in survivors without BPD_{28d} was 13.5 pg/mL (6, 30), $p = 0.12$. Therefore, it is unlikely that the association of bFGF with BPD or death is the result of its correlation with birth weight or gestational age.

bFGF levels were not significantly different in infants after chorioamnionitis *versus* those not exposed to chorioamnionitis (Table 2). bFGF levels correlated well with apoptosis (Spearman's $r = 0.73$, $p < 0.001$) and LDH levels ($r = 0.59$, $p < 0.001$) (Fig. 1). On the other hand, apoptosis and LDH levels did not correlate well with each other (Spearman's $r = 0.27$, $p = 0.16$).

VEGF. VEGF levels were not significantly different in infants who survived without BPD_{28d} *versus* those who died or developed BPD_{28d} (Table 1). VEGF levels in infants who had BPD_{36wk} or died [105 pg/mL (41, 315)] were also not significantly different from normal survivors [165 pg/mL (15, 480); $p = 0.60$]. VEGF levels showed a statistically nonsignificant trend to be higher in infants after chorioamnionitis *versus* those not exposed to chorioamnionitis (Table 2). There was no significant correlation between VEGF levels and birth weight (Spearman's $r = 0.19$, $p = 0.33$) or gestational age (Spearman's $r = 0.16$, $p = 0.41$). VEGF did not also correlate with LDH levels (Spearman's $r = -0.11$, $p = 0.6$) or apoptosis (Spearman's $r = -0.28$, $p = 0.13$).

ET-1. ET-1 levels were not significantly different in infants who survived without BPD_{28d} *versus* those who died or developed BPD_{28d} (Table 1). ET-1 levels in infants who had BPD_{36wk} or died [90 pg/mL (49, 110)] were also not significantly different from normal survivors [66 pg/mL (45, 116); $p = 0.60$]. ET-1 levels were also not different in infants after chorioamnionitis *versus* those not exposed to chorioamnionitis (Table 2). There was no significant correlation between ET-1

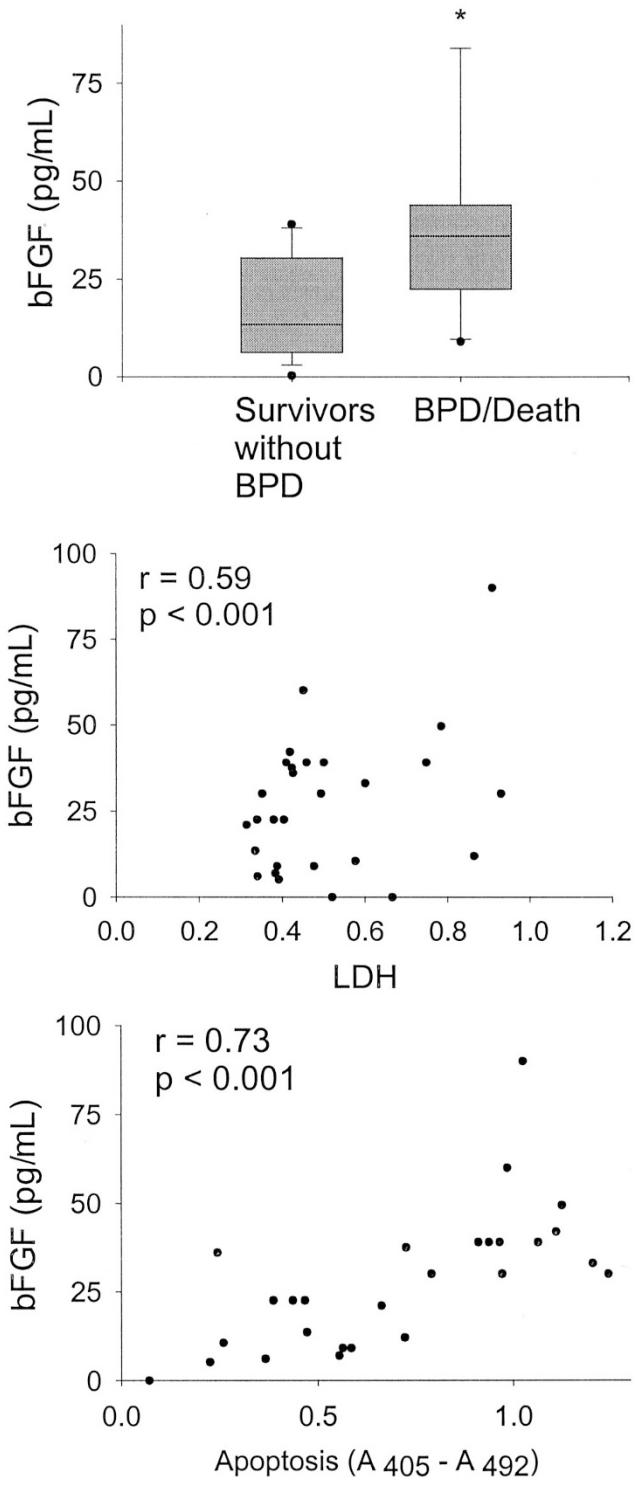

Figure 1. *Top panel* is a box-plot of tracheal aspirate bFGF in relation to the outcome of BPD_{28d}/death. The *central line* in the box indicates the median value and the top and bottom of the box indicate the 75th and 25th centiles, respectively. The *whiskers* indicate the 10th and 90th centiles, and the additional *dark circles* show the outliers. The *middle panel* is a scatter-plot of bFGF *vs* LDH levels, and the lower panel is a scatter-plot of bFGF in relation to apoptosis levels. (*$p < 0.05$ *vs* other group). One outlier has been removed from all panels.

Table 2. *PGF levels in tracheal aspirates of mechanically ventilated preterm infants in relation to antecedent chorioamnionitis*

PGF	No chorioamnionitis (n = 11)	Chorioamnionitis (n = 18)	p Value*
bFGF	22.5 (15, 39)	30 (9, 39)	.81
VEGF	96 (4, 148)	323 (105, 600)	.06
ET-1	66 (39, 92)	93 (57, 135)	.27

Values given in picograms per milliliter [mean (25%, 75%)].
* Mann-Whitney rank sum test.

levels and birth weight (Spearman's $r = -0.14$, $p = 0.48$) or gestational age (Spearman's $r = -0.19$, $p = 0.32$). ET-1 weakly correlated with apoptosis (Spearman's $r = 0.38$, $p = 0.04$) but not with LDH levels (Spearman's $r = 0.29$, $p = 0.13$).

Cells in tracheal aspirate. Airway epithelial cells and neutrophils were the predominant cell types observed. No correlation was present between levels of bFGF, VEGF, and ET-1 *versus* cell density, proportion of the cells that were WBC, proportion of the WBC that were neutrophils, and the absolute number of neutrophils/high power field (hpf) (data not shown; all $r < 0.40$, all $p > 0.05$). No correlation was present between the presence or absence of chorioamnionitis or poor outcome (death/BPD_{28d}) and the cell types or numbers observed in the tracheal aspirate (mean ± SD; death/BPD_{28d}: 74 ± 74 cells/hpf, 27 ± 21% WBC, 63 ± 30% neutrophils, 15 ± 25 neutrophils/hpf *versus* normal survivors: 71 ± 46 cells/hpf, 42 ± 23% WBC, 72 ± 11% neutrophils, 17 ± 13 neutrophils/hpf).

DISCUSSION

This pilot study evaluated the association between PGF in tracheal aspirates of mechanically ventilated preterm infants and BPD, markers of lung injury, and antecedent chorioamnionitis. Elevated levels of bFGF in tracheal aspirates on the first day of life were associated with worse outcome (BPD_{28d}/death) and increased evidence of cell injury (LDH) and apoptosis (DNA fragmentation). VEGF and ET-1 did not demonstrate such an association, and all three PGF were not linked to chorioamnionitis.

Our study has some important limitations. Being a pilot study, the sample size was limited, increasing the likelihood of statistical error. Subsequent trials using a larger sample size are required to confirm these results. The sample size was limited as our center uses conservative indications for the initiation of mechanical ventilation in preterm neonates, and rapid weaning from the ventilator is the norm. Endotracheal suctioning is also not routinely performed on the first day of life unless there are excessive secretions or suspicion of tube occlusion. The study population is hence a higher-risk category of ventilated preterm infants. It is controversial whether tracheal aspirates should be corrected for dilution, using techniques such as urea dilution, protein levels, or secretory immunoglobulin A (SIgA) concentrations. We followed the recommendations of the European Respiratory Task Force on Bronchoalveolar Lavage in children (19) and did not correct our results for the dilution, although our procedure could be more accurately defined as tracheobronchial lavage rather than bronchoalveolar lavage. Increased vascular permeability and lung inflammation in sicker infants may lead to erroneous results after correction for urea dilution or protein concentration, as increased levels of urea or protein may not signify less dilution but a leakier air-epithelial interface. Another limitation of this and similar studies is that the origin of growth factors and other substances present in the tracheal aspirates cannot be determined with certainty.

There are some possible explanations for the association between higher bFGF and worse outcome. More research is required to evaluate the role and implications of bFGF in the preterm airway, as this is the first report to address the issue. Fibroproliferation is initiated early (within 24 h of diagnosis) in ARDS and impacts on outcome (20). bFGF is elevated in the epithelial lining fluid of adults with ARDS, but not in those without acute lung inflammation (18). It is possible that a similar process occurs in neonatal respiratory distress syndrome, and that bFGF is one of the stimuli inducing fibroproliferation. The association between bFGF and outcomes or markers of cell injury/apoptosis in preterm infants has not been described so far. Basic FGF is localized to airway epithelial cells and extracellular matrix in rat fetal lungs (1), and in both epithelial and mesenchymal cells in human fetuses (2). bFGF may modulate repair from lung injury (21, 22). The mechanism of bFGF release is unclear, as it lacks a signal sequence (although higher molecular weight forms contain a nuclear targeting sequence). It is likely that cell injury and inflammation lead to a release of bFGF with autocrine effects. This is in agreement with our observation that elevated levels of bFGF correlated with LDH and apoptosis markers, suggesting that cellular injury and apoptosis may lead to the bFGF release. We speculate that the cellular injury and apoptosis in combination with elevated bFGF may either indicate sufficient lung injury to cause mortality or BPD_{28d}, or may directly contribute at least in part to these outcomes. It is possible that tissue concentrations of bFGF are higher, compared with levels in the diluted tracheal aspirate, and may co-localize with sites of injury and repair processes.

VEGF is an endothelial cell mitogen that also regulates vascular permeability. VEGF is found mostly in bronchial epithelium and alveolar macrophages, and its receptor (Flt-1) is found in vascular endothelial cells and bronchial epithelial cells (14). Infants dying from BPD have dysmorphic alveolar capillaries in association with decreased VEGF mRNA and immunostaining, and decreased message for Flt-1 and the angiopoietin-1 receptor TIE-2 (17). Lassus *et al.* (23) observed a rapid postnatal increase in VEGF levels in tracheal aspirates from intubated preterm infants, without any correlation between VEGF and birth weight or gestational age. During d 4 and 7, infants developing BPD_{36wk} had lower VEGF than survivors without BPD (23). D'Angio *et al.* (24) noted a tripling of VEGF levels in BAL fluid between d 1 and 3, and higher lavage levels on d 1 and 3 correlated with a lower gestational age, but not with the development of BPD_{36wk}. Our study is in concurrence with that of Lassus *et al.* (14) and Bhatt *et al.* (17), as infants with worse outcome (BPD_{28d} or death) had a nonsignificant trend toward lower VEGF levels (median

level of 114 pg/mL *versus* 290 pg/mL in survivors without BPD_{28d}, $p = 0.13$). It must be emphasized that all studies to date, including ours, had small sample sizes and did not have sufficient statistical power to arrive at definite conclusions.

ET-1 is developmentally regulated (25) and is involved in the pathogenesis of related disorders such as ARDS (26). Niu *et al.* (15) observed in preterm infants that tracheal aspirate ET-1 was elevated in the first day in infants who later developed BPD_{36wk}. However, in a study on 11 preterm neonates in the first week of life, Andersson *et al.* (27) concluded that higher ET-1 levels in tracheal aspirates were associated with lower mean airway pressures and oxygen concentration (Fio_2). Andersson *et al.* (27) did not find any correlation between ET-1 and gestational age and birth weight. Our study did not show any associations between ET-1 and outcome, or between ET-1 and birth weight and gestational age.

Other PGF that have not been evaluated in this study, such as EGF, transforming growth factor-α, PDGF, and KGF (FGF-7), are also known to be important *in vivo* in normal lung development in late fetal life and in repair from injury (8, 28–33).

Chorioamnionitis is a risk factor for poor outcome in preterm neonates (34, 35), and we have shown earlier that chorioamnionitis was associated with elevations of proinflammatory cytokines (IL-1β, IL-6, IL-8, tumor necrosis factor-α) (36). The high rate of BPD_{28d}/death in this study may have been the result of the high incidence of chorioamnionitis (18 of 29 infants), which is known to predispose to BPD and mortality. However, the levels of bFGF, VEGF, and ET-1 were similar in infants with or without chorioamnionitis, suggesting that these growth factors may not have been involved in chorioamnionitis-induced lung pathology on the first day of life.

A larger study is required to confirm the association of bFGF with worse outcomes, especially with longer-term outcomes such as BPD_{36wk}, pulmonary function in early childhood, and neurodevelopment. Basic research is necessary to determine whether the association is of a causal nature, and, if so, evaluate the possible therapies that may exploit this mechanism of disease.

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