Cord Blood Angiogenic Progenitor Cells Are Decreased in Bronchopulmonary Dysplasia

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Sources of Support: National Institutes of Health (K12 HL090147-01 PI: M Geraci, Awardee: CDB; R01 HL089262 PI: VB; NCRR 5K23RR021921 PI: PMM; HL085703-01 PI: SHA; R01 HL68702 PI: SHA; Colorado CTSI 5UL1 RR025780); Thrasher Foundation (PI: SHA)

Running Title: EPCs Are Decreased in BPD

Word Count: 2748

Tables: 1    Figures: 3

This article has online supplemental materials.

Abbreviations:
BPD: bronchopulmonary dysplasia
BW: birth weight
CPAP: continuous positive airway pressure
CPC: circulating progenitor cell
ECFC: endothelial colony-forming cell
EPC: endothelial progenitor cell
GA: gestational age
IQR: interquartile range
IUGR: intrauterine growth restriction
MNC: mononuclear cell
MSC: mesenchymal stromal cell
MV: mechanical ventilation
OFC: occipitofrontal circumference
PFC: polychromatic flow cytometry
ROP: retinopathy of prematurity
SD: standard deviation
VEGF: vascular endothelial growth factor

Published on April 10, 2012 as doi: 10.1183/09031936.00017312

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Abstract
Bronchopulmonary dysplasia (BPD), the chronic lung disease of prematurity, is associated with impaired vascular and alveolar growth. Antenatal factors contribute to the risk for developing BPD by unclear mechanisms. Endothelial progenitor cells (EPCs), such as angiogenic circulating progenitor cells (CPCs) and late-outgrowth endothelial colony-forming cells (ECFCs), may contribute to angiogenesis in the developing lung. We hypothesize that cord blood angiogenic CPCs and ECFCs are decreased in preterm infants with moderate and severe BPD.

We quantified ECFCs and the CPC-to-nonangiogenic-CPC ratio (CPC:non-CPC) in cord blood samples from 62 preterm infants and assessed their relationships to maternal and perinatal risk factors as well as BPD severity. The CPC:non-CPC ratio and ECFC number were compared between preterm infants with mild or no BPD and those with moderate or severe BPD.

ECFC number (p < 0.001) and CPC:non-CPC ratio (p < 0.05) were significantly decreased in cord blood samples of preterm infants who subsequently developed moderate or severe BPD. Gestational age and birth weight were not associated with either angiogenic marker.

Circulating vascular progenitor cells are decreased in the cord blood of preterm infants who develop moderate and severe BPD. These findings suggest that prenatal factors contribute to late respiratory outcomes in preterm infants.

Abstract Word Count: 200
Bronchopulmonary dysplasia (BPD), the chronic lung disease of prematurity, was originally described as the result of postnatal lung injury from mechanical ventilation and oxygen supplementation [1]. While advances in the treatment of neonatal respiratory distress syndrome have improved the survival of extremely low birth weight infants, BPD remains the most common complication of preterm birth [2]. In the post-surfactant era, the “new BPD” is characterized by a developmental arrest of lung vascular and alveolar growth resulting in decreased surface area for gas exchange [2-4] .BPD is associated with increased mortality and morbidities such as respiratory insufficiency, chronic hypoxemia, pulmonary hypertension, exercise intolerance, wheezing, and poor neurodevelopmental outcomes [5].

The extent of chronic lung disease is highly variable among preterm infants. While gestational age (GA) and birth weight (BW) are the most predictive indicators of BPD severity, antenatal factors such as preeclampsia, chorioamnionitis, and intrauterine growth restriction (IUGR) contribute significantly to the pathogenesis of “new BPD” [2, 6]. The “Barker Hypothesis” suggests that intrauterine factors contribute to the risk for respiratory disease later in life [7]. However, little is known about underlying mechanisms by which antenatal events contribute to impaired lung development and function. Postnatal factors such as high levels of supplemental oxygen, patent ductus arteriosus, infection, prolonged mechanical ventilation, and malnutrition, are also associated with more severe respiratory outcomes [8]. A subset of infants develops BPD, but the presence or absence of postnatal factors alone may not be sufficient to determine the risk for chronic lung disease in preterm infants.
Both human and animal studies suggest that impaired vascular growth plays a central role in the pathogenesis of BPD [3, 9, 10]. Vascular endothelial growth factor (VEGF) is decreased in the lungs of human infants dying from severe BPD [9]. Anti-angiogenic mediators such as endostatin have been associated with the development of BPD [11]. In preeclampsia, a known contributor to BPD risk, soluble-Flt1 (another potent anti-angiogenic factor) is increased [12].

Vascular growth occurs by either angiogenesis (the direct extension of existing vessels) or vasculogenesis [13]. In vasculogenesis, new vessels are formed from primitive hemangioblasts located within a developing organ or from putative bone marrow-derived circulating endothelial progenitor cells (EPCs) that are thought to home to developing microcapillary beds in the lung and other organ systems [13, 14].

EPCs are decreased in the blood, lungs, and bone marrow of newborn mice with experimental BPD due to hyperoxia [15]. This suggests that postnatal events can disrupt vasculogenic mechanisms during development leading to impaired lung vascular and alveolar growth. Systemic and intra-tracheal administration of mesenchymal stromal cells (MSCs) or MSC-conditioned media to neonatal rodents partially restores lung architecture during neonatal hyperoxia [16, 17]. In addition, intravenous administration of bone marrow-derived proangiogenic cells after exposure to neonatal hyperoxia normalizes lung structure in infant mice [18].

Based on these data from preclinical models of BPD, we speculate that angiogenic progenitor levels in umbilical cord blood samples at birth may be inversely associated with the severity of BPD. More specifically, we hypothesize that preterm infants who have decreased levels of
angiogenic progenitors in their cord blood will have an increased risk for developing severe BPD. In this prospective cohort study, we report that preterm infants who later develop moderate and severe BPD have decreased angiogenic progenitor cells in umbilical cord blood, as reflected by reduced late-outgrowth endothelial colony-forming cells (ECFCs) and by the ratio of pro-angiogenic circulating progenitor cells (CPCs) to non-angiogenic CPCs (CPC:non-CPC).

Methods

Subject enrollment and data collection
The Colorado Multiple Institutional Review Board approved all study protocols. Informed consent was obtained from pregnant mothers presenting in preterm labor. Subjects were enrolled from October 2009 until June 2011. Cord blood was collected and processed as previously described [19]. Eligible mothers were admitted to the University Hospital Anschutz Inpatient Pavilion, delivering a newborn of 24-36 weeks GA, and capable of providing consent. Exclusion criteria included known HIV/HBV/HCV infection. Maternal and infant clinical data were collected until hospital discharge. A clinical diagnosis of chorioamnionitis was made in the setting of uterine tenderness and maternal fever. IUGR was defined as BW less than the 10th percentile with an occipitofrontal circumference greater than the 10th percentile. Data were stored in a secure REDCap database [20].

Determination of BPD status
The presence and severity of BPD were determined using NIH criteria with an adjustment for the local altitude of 1600m (Figure S1) [8]. An oxygen reduction test (see Supplemental Material)
was performed when indicated. Briefly, the diagnosis of BPD was made at 36 weeks post-conception (or day of life 28 if the infant was born after 32 weeks). Infants who required supplemental oxygen for less than 28 days, had no BPD. Those infants with an FiO₂ of less than 0.26 had mild BPD. If the FiO₂ was between 0.26 and 0.35, the infant had moderate BPD. Finally, severe BPD indicates that the child required an FiO₂ of greater than 0.35 or positive pressure (mechanical ventilation, CPAP, high flow oxygen by nasal cannula). Subjects were separated into two study groups, those with mild or no BPD and those with moderate or severe BPD, based on the rationale that at sea level infants with mild or no BPD do not require supplemental oxygen at the time of diagnosis.

**Cord blood collection and ECFC isolation**

Samples were maintained at room temperature and analyzed within 24 hours. Mononuclear cells (MNCs) were isolated by gradient centrifugation for both the ECFC culture assay and polychromatic flow cytometry (PFC). MNCs were cultured on type 1 collagen in complete EGM-2 media (Lonza) with 10% fetal bovine serum. ECFC colonies (Figure S2A) were identified daily and enumerated on day 14. Low-passage (p2-3) ECFCs were characterized by immunohistochemistry, PFC, tube formation, and a single-cell assay to confirm self-renewal (Figure S2B-E).

**Polychromatic flow cytometry**

MNCs (Figure S3A) were analyzed by PFC as follows. 0.5-1.0 x 10⁶ cells were stained with antibodies to CD31, CD34, CD45, AC133, Glycophorin-A (erythrocyte exclusion), CD14 (macrophage exclusion), and a LIVE/DEAD viability marker. Compensation beads, fluorescence
minus-one controls, and bi-exponential gating were utilized to facilitate accurate compensation and gating [21]. Angiogenic (CD45<sup>dim</sup>CD34<sup>+</sup>CD31<sup>+</sup>AC133<sup>+</sup>) and non-angiogenic (CD45<sup>dim</sup>CD34<sup>-</sup>CD31<sup>+</sup>AC133<sup>-</sup>) CPCs were measured so that the CPC:non-CPC ratio could be determined using established methods (Figure S3B-C) [21, 22]. PFC analysis was performed using a CyAn 9-color flow cytometer (Beckman Coulter) and FlowJo software (v. 9.3.2).

**Statistical Analysis**

Non-parametric data were analyzed using Mann Whitney tests and are presented as medians with interquartile ranges (IQR). Normally distributed data were analyzed using unpaired t-tests and are presented as means with standard deviations (SD). Spearman’s correlation coefficients were utilized to compare ECFC number with GA and BW. Fisher’s exact test was used to analyze categorical data. Analysis was performed with the Prism software package (v. 5.0, GraphPad). Significance level was set at $\alpha = 0.05$. The first author and biostatistician (MKS) analyzed all data.

**Results**

**Patient characteristics**

As summarized in Table 1, 62 preterm infants (24-36 weeks) were enrolled. 13 (21.0%) developed moderate or severe BPD. The remaining 49 (79.0%) developed none or mild BPD. In comparison with infants who had mild or no BPD, GA, BW, length, and head circumference were significantly decreased in patients with moderate or severe BPD (Table 1). Ventilator days, CPAP days, CPAP plus ventilator days, and total days with supplemental oxygen were greater in
the more severe group. The incidence of preeclampsia, chorioamnionitis, maternal diabetes, and smoking were similar between groups. Apgar scores at one and five minutes were greater in the mild or no BPD group.

**Angiogenic circulating progenitor cells are decreased in moderate and severe BPD**

PFC was performed on the cord blood of 60 of the 62 subjects. 12 samples were excluded because fluorochrome oversaturation prevented CPC enumeration. Of the remaining 48, 37 had mild or no BPD and 11 had moderate or severe BPD. The CPC:non-CPC ratio was significantly lower in the cord blood of infants who developed moderate or severe BPD (1.5 [1.2, 1.8] vs. 2.5 [1.5, 3.9]; p < 0.05; Fig 1A). When samples from all infants who later developed BPD (n=29) were compared to those who did not (n=19), the CPC:non-CPC ratio trended lower in those with BPD, but this failed to achieve statistical significance (1.8 [1.3, 3.0] vs. 2.8 [1.8, 4.9]; p = 0.07; Fig S4A). In the subset of infants born at or before 28 weeks (n=11), the trend towards a decreased CPC:non-CPC ratio was not statistically significant (Fig S4B).

**Cord blood ECFCs are decreased in moderate and severe BPD**

Cord blood ECFC number was markedly decreased in preterm infants who went on to develop moderate or severe BPD as compared to those who did not (0 [0, 0.8] vs. 3.6 [0.9, 8.0] colonies per 10^7 MNCs; p < 0.001; Fig 1B). ECFCs were also decreased among all subjects with BPD (n=36) as compared to those without BPD (n=26; 1.4 [0, 4.8] vs. 3.9 [1.3, 7.4]; p < 0.05; Fig 1C). Among infants born at or before 28 weeks, all of these infants developed BPD. However, none of the infants with moderate or severe BPD had detectable ECFCs in their cord blood. Infants with mild BPD had 4.9 [2.4, 9.0] ECFC colonies per 10^7 MNCs (p < 0.01; Fig 1D).
By univariate non-parametric comparison, neither GA (r = 0.23; p = 0.11) nor BW (r = 0.24; p = 0.11) affected the CPC:non-CPC ratio. In contrast to previous studies, we found that GA did not correlate with cord blood ECFC number (r = 0.17; p = 0.18; Fig 2A).[23, 24] Although a nonparametric comparison of ECFC number with BW was statistically significant, the coefficient of determination shows that the correlation is small (r = 0.26; p = 0.04; Fig S4C).

A small but significant correlation was found between the CPC:non-CPC ratio and ECFC number (r = 0.33; p = 0.02; Fig S4D). We then compared samples that contained ECFCs (n=32) to those that did not (n=16) and found that the CPC:non-CPC ratio was significantly higher in ECFC-containing cord blood (2.8 [1.8, 4.2] vs. 1.5 [1.1, 1.8]; p = 0.001; Fig 2B).

**Association of perinatal risk factors and cord blood ECFC number**

Although its incidence was no different between groups, we found that infants with clinical chorioamnionitis had increased cord blood ECFCs(n=10; 8.0 [3.9, 8.5] vs. 1.8 [0, 4.7]; p < 0.01; Fig 3A). In the present study, cord blood ECFCs tended to be lower in maternal preeclampsia (n=13), but the difference was not statistically significant (1.0 [0, 4.7] vs. 3.0 [0, 7.0]; p = 0.23; Fig S4E). Birth by Caesarean section (n=30) resulted in a significantly decreased cord blood ECFC number as compared to vaginal birth (1.1 [0, 3.5] vs. 5.0 [1.0, 8.0]; p < 0.01; Fig 3B). There was no difference between planned and emergent Caesarean delivery (not shown).

Antenatal corticosteroid treatment did not alter ECFC number (Fig S4F). None of these antenatal factors significantly affected the CPC:non-CPC ratio.
Discussion

We report that circulating angiogenic progenitor cells, including late outgrowth ECFCs and the CPC:non-CPC ratio, are decreased in the cord blood of preterm infants who develop moderate or severe BPD. In this population, neither the CPC:non-CPC ratio nor ECFC number are strongly associated with prematurity, as determined by GA or BW. Reduction in both angiogenic markers was associated with increased BPD. Preterm newborns with chorioamnionitis have increased cord blood ECFCs and infants delivered via Caesarean section have decreased ECFCs. While enrollment was not sufficient to demonstrate a significant difference, preterm infants born to mothers with preeclampsia tended to have decreased cord blood ECFCs.

A recent epidemiologic study supported “Barker’s Hypothesis” by demonstrating links between antenatal events and late respiratory outcomes such as BPD [6]. However, Hansen and colleagues noted that only 54% of the odds variability for developing BPD could be described using the occurrence of preeclampsia, clinical chorioamnionitis, male sex, and maternal smoking as well as gestational age and birth weight z-score [6]. Our findings are interesting in that cord blood ECFC and CPC:non-CPC levels are low in preterm infants who subsequently develop moderate or severe BPD. These data directly support the concept that antenatal events affect late respiratory outcomes in preterm infants, and suggest that EPCs may serve as potential biomarkers to better identify at risk infants. However, whether altered EPC levels or function directly contribute to the pathobiology of BPD remains unknown.

Although first isolated over ten years ago, much remains unknown about the role of EPCs in vascular growth during fetal development. EPCs have been isolated and quantified by both PFC
and primary culture assays [25, 26]. Cultured EPCs are increased in human disease states such as acute lung injury in which early-outgrowth EPCs correlate with survival [27]. Although early-outgrowth EPCs augment angiogenesis, these cells do not have an endothelial morphology and function as angiogenic macrophages [28, 29]. In comparison, late-outgrowth ECFCs have an endothelial appearance, are stem-like (highly-proliferative and self-renewing), and form chimeric vessels in vivo [26, 29]. However, ECFCs may have fewer paracrine effects on angiogenesis [28]. Both early EPCs and ECFCs may be required for effective angiogenesis in the developing lung.

EPCs were first described by PFC as CD34⁺AC133⁺KDR⁺ cells [14, 30]. However, we did not measure these cells as they are not different in the cord blood of full-term infants and preterm infants whether or not they later develop BPD [19, 31]. In contrast, CPCs express a different surface antigen profile (CD45dimCD34⁻CD31⁺AC133⁺), are proangiogenic, and promote tumor growth in vivo [22]. Therefore, we included a CD31 antibody in our staining protocol in lieu of identifying KDR-positive cells. Non-CPCs are AC133-negative (CD45dimCD34⁻CD31⁺AC133⁻) and are not angiogenic [22]. The CPC:non-CPC ratio is decreased in peripheral artery disease and gestational diabetes, but its significance in the cord blood of preterm infants with BPD has not been previously described [22, 32]. While the absolute number of circulating CPCs may be relevant, there was no difference in CPCs between study groups. The CPC:non-CPC ratio may more accurately reflect the balance between competing mechanisms the body uses to regulate vascular growth.
A previous study suggested that ECFCs may be decreased in a small subset of patients with BPD, but whether these findings simply reflect the association between the severity of BPD and degree of prematurity was unclear [23]. We did not observe ECFC number to be a function of GA or BW, as previously reported in two smaller studies, suggesting that the link between ECFCs and late outcomes are not simply due to an association with the degree of prematurity [23, 24]. Circulating progenitors may directly contribute to pulmonary angiogenesis [10, 15, 18] and we speculate that decreased ECFCs may contribute to abnormal vascular growth and more severe BPD. Further study is needed to better elucidate the role of CPCs and ECFCs during normal vascular development and how changes in EPC number or function contributes to BPD.

Cord blood ECFCs are increased with chorioamnionitis and vaginal birth. We speculate that these processes result in significant perinatal stress that causes a release of angiogenic progenitors into the circulation. We note that the CPC:non-CPC ratio was not similarly increased in these infants. Further study is needed to confirm these findings and to identify the angiogenic factors that mediate this response.

The small number of patients enrolled in the study may result in type II errors and limits our ability to perform multivariate analysis in depth. These findings neither confirm nor refute the hypothesis that decreased circulating EPCs actually contribute to impaired vascular growth in BPD. Nevertheless, the striking association between decreased EPCs and BPD severity leads us to speculate that disruption of pro-angiogenic precursor cells are involved in the pathogenesis of BPD.
We conclude that angiogenic CPCs and ECFCs, potential cellular biomarkers of angiogenic activity, are decreased in the cord blood of preterm infants who go on to develop moderate or severe BPD. Using the data presented, we determined that a CPC:non-CPC ratio of less than two and the absence of cord blood ECFCs convey relative risks of 5.2 (95% CI: 1.2, 21.9) and 8.1 (95% CI: 2.5, 26.2), respectively. However, further large-scale trials including validation cohorts are needed to confirm if either of these assays provides biomarkers that predict which infants are at greatest risk for chronic lung disease. We speculate that antenatal factors, genetic predisposition, or both, decrease circulating EPCs in the fetal circulation, which may impair postnatal vasculogenesis and contribute to the severity of late respiratory outcomes after preterm birth. A better understanding of the function of both CPCs and ECFCs will lead to novel therapies to promote pulmonary vascular growth in the preterm newborn and improve outcomes in this high-risk neonatal population.

Support Statement
This study was funded by the National Institutes of Health (K12 HL090147-01 PI: M Geraci, Awardee: CDB; R01 HL089262 PI: VB; NCRR 5K23RR021921 PI: PMM; HL085703-01 PI: SHA; R01 HL68702 PI: SHA; Colorado CTSI 5UL1 RR025780) and the Thrasher Foundation (PI: SHA).

Contributors
Study concept/design – all authors. Data acquisition – CDB, CPB, SLR. Statistical analysis – CDB, VB, MKS, SHA. Manuscript first draft – CDB. Manuscript revisions – CDB, VB, PMM, MKS, SHA. Approval of final manuscript – all authors.
Statement of Interest

All authors have completed a statement of interest. No potential conflicts are reported.

Acknowledgements

References


Figure Legends

Figure 1. Angiogenic progenitor levels in preterm newborns by BPD severity. (A) The ratio of angiogenic CPCs to non-angiogenic CPCs (CPC:non-CPC) is significantly greater in the cord blood of infants with mild or no BPD (n=37; solid circles) as compared to those with moderate or severe BPD (n=11; open circles; * p < 0.05). (B) Cord blood ECFC number, defined as the number of ECFC colonies on day 14 per 10^7 MNCs plated, is significantly greater in infants who later developed mild or no BPD (n=49; solid circles) as compared to those infants who developed moderate or severe BPD (n=13; open circles; *** p = 0.001). (C) Cord blood ECFC number is greater in infants with no BPD (n=26; solid circles) as compared to those with BPD of any severity (n=36; open circles; * p < 0.05). Among infants born at ≤ 28 weeks, (D) Cord blood ECFC number was significantly decreased in infants with moderate or severe BPD (n=5; open circles) as compared to those with mild BPD (n=9; solid circles; ** p < 0.01).

Figure 2. Cord blood ECFC number (on day 14) plotted against gestational age and the CPC:non-CPC ratio. (A) There is no correlation between cord blood ECFC number and gestational age (Spearman r = 0.17; p = 0.18). (B) In infants whose cord blood yielded no ECFC colonies (open circles), the CPC:non-CPC ratio was significantly decreased as compared to those with cord blood ECFCs (closed circles; *** p < 0.001).

Figure 3. The influence of prenatal factors on cord blood ECFC number. (A) ECFCs are increased in the cord blood of infants born with clinical chorioamnionitis (n=10; open circles) as compared to those without chorioamnionitis (n=50; solid circles; ** p < 0.01). (B) Infants born
via Caesarean section (n=30; open circles) had significantly fewer cord blood ECFCs than those born vaginally (n=32; closed circles; ** p < 0.01)
### Table 1. Clinical characteristics of the newborn infants.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>None-Mild BPD</th>
<th>Mod-Severe BPD</th>
<th>p-value</th>
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<tr>
<td>Total Subjects</td>
<td>49</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>26/23</td>
<td>9/4</td>
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<td>Born before 28 weeks - %, n</td>
<td>18.4, 9</td>
<td>38.5, 5</td>
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<td>32.1, 3.1</td>
<td>29.7, 3.2</td>
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<td>Birth Weight - g (mean, SD)</td>
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<td>1256, 488</td>
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<td>Length - cm (mean, SD)</td>
<td>42.4, 4.5</td>
<td>38.0, 6.1</td>
<td>0.005</td>
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<td>OFC - cm (mean, SD)</td>
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<td>27.0, 3.2</td>
<td>0.03</td>
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<td>Maternal Smoking - %, n</td>
<td>8.2, 4</td>
<td>15.4, 2</td>
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<td>Preeclampsia - %, n</td>
<td>20.4, 10</td>
<td>23.1, 3</td>
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<tr>
<td>Diabetes - %, n</td>
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<td>Prenatal Corticosteroids - %, n</td>
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<td>90.9, 10</td>
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<td>Caesarian Section - %, n</td>
<td>44.9, 22</td>
<td>61.5, 8</td>
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<td>76.9, 10</td>
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<td>Chorioamnionitis - %, n</td>
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<td>Apgar 5 min (median, IQR)</td>
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<td>Vent days (median, IQR)</td>
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<td>CPAP days (median, IQR)</td>
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<td>Total O2 days (median, IQR)</td>
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<td>ROP (abnormal exam)- %, n</td>
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<td>69.2, 9</td>
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<td>ROP (laser treatment)- %, n</td>
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**Patient characteristics.** Definitions of abbreviations: n = number of subjects within a study group with the given condition; BPD = bronchopulmonary dysplasia; OFC = occipitofrontal circumference; CPAP = continuous positive airway pressure; O2 = supplemental oxygen; ROP = retinopathy of prematurity; SD = standard deviation; IQR = interquartile range.
FIGURES

Fig 1A

![Graph showing CPC:non-CPC ratio for None-Mild BPD (n=37) and Mod-Sev BPD (n=11).](image)
Fig 1B

![Graph showing ECFCs/10^7 MNCs for None-Mild BPD (n=49) and Mod-Sev BPD (n=13). The graph displays a significant difference between the groups indicated by the label ***]
Fig 2A

ECFCs / $10^7$ MNCS vs. Gestational Age (weeks)

$r = 0.17$
Fig 2B

CPC:non-CPC ratio

ECFCs
n=32

No ECFCs
n=16

***