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Low Urine Vascular Endothelial Growth Factor Levels Are Associated with Mechanical Ventilation, Bronchopulmonary Dysplasia and Retinopathy of Prematurity

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Key Words

Vascular endothelial growth factor · Bronchopulmonary dysplasia · Retinopathy of prematurity · Mechanical ventilation · Fraction of inspired oxygen · Enzyme-linked immunosorbent assay · Biomarker

Abstract

Background: Organ-specific vascular endothelial growth factor (VEGF) expression is decreased during the pathogenesis of bronchopulmonary dysplasia (BPD) and retinopathy of prematurity (ROP) several weeks before either disease can be diagnosed. Early measurement of organ-specific tissue VEGF levels might allow identification of infants at high risk for these diseases, but is not clinically feasible. Urine VEGF is easily measured and useful in early diagnosis of several diseases. ***Objectives:*** Our aims were to assess the correlation of urine VEGF levels measured in the first postnatal month with subsequent BPD or ROP diagnosis and to determine whether various infant characteristics influence urine VEGF levels. ***Methods:*** 106 subjects born at <29 weeks' gestation and surviving to 36 weeks' postmenstrual age were selected from an existing database and biorepository. Urine VEGF and total protein were measured in 2–3 samples per subject. ***Results:*** Urine VEGF/protein levels increased by 72% per week ($p < 0.0001$) during the first postnatal month. In multivariable analysis controlling for postnatal age, lower VEGF/protein was associated with higher levels of mechanical respiratory support ($p = 0.006$), male gender ($p = 0.001$) and early sepsis ($p = 0.003$) but not with fraction of inspired oxygen. Lower urine VEGF/protein and mechanical ventilation were each associated with BPD and ROP. In analyses adjusted for respiratory support, lower urine VEGF/protein and ROP remained associated but urine VEGF/protein and BPD did not. ***Conclusions:*** Low urine VEGF/protein levels in the first postnatal month are associated with mechanical ventilation, BPD, and ROP.

Introduction

Bronchopulmonary dysplasia (BPD) and retinopathy of prematurity (ROP) are common complications of prematurity that result from abnormal postnatal development of the lung and eye, respectively. While the developmental programs of each organ are unique, both are critically de-

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pendent on angiogenesis, and disruption of normal angiogenesis is central to the pathogenesis of both diseases.

BPD is a chronic lung disease characterized by alveolar simplification and capillary dysplasia [1] that affects 42% of infants born before 29 weeks' gestation [2]. BPD results, in part, from failure of postnatal secondary septation, a morphogenic process that utilizes angiogenesis to subdivide the saccular airspaces of the immature lung into alveoli [1]. Lung angiogenesis is largely regulated by vascular endothelial growth factor (VEGF) [3], and VEGF expression is suppressed in BPD [4, 5]. While the pathogenesis of BPD is multifactorial, augmenting VEGF signaling and angiogenesis can prevent BPD in animal models [6–8] and might do the same in human infants.

ROP is a neoproliferative retinopathy caused by abnormal postnatal development of the retina that affects 59% of infants born before 29 weeks' gestation [2]. Retinal blood vessels normally develop by VEGF-driven angiogenesis [9]. In early stages of ROP, VEGF is suppressed and the retinal vessels stop growing [10]. In later stages, hypoxia of the avascular retina triggers VEGF overexpression and proliferative neovascularization [9]. Current treatments reduce VEGF signaling during late ROP [11], but augmenting VEGF expression in early ROP can prevent both the loss of retinal vessels and subsequent neoproliferation in animal models [10, 12] and has been proposed as a preventative treatment for human infants [9].

Both BPD and ROP are characterized by VEGF suppression after preterm birth and afflict the same population of infants. Each might be prevented by early VEGF augmentation. We hypothesized that urine VEGF levels are lower in the first postnatal month among premature infants who later develop BPD or ROP. Our study aims were to assess the association between urine VEGF and subsequent diagnosis of BPD and/or ROP and to evaluate the impact of various subject characteristics on urine VEGF levels.

Materials and Methods

Study Population

Subjects were selected from the Boston Children's Hospital Specialized Centers of Research (SCOR) in lung biology database of infants born before 29 weeks' gestation. Eligible subjects were born at Brigham and Women's Hospital (BWH), survived to 36 weeks' postmenstrual age (PMA), and had ≥2 urine samples of adequate volume separated by ≥4 days obtained during the first postnatal month. Our goal was to enroll ≥100 subjects and process ≥200 urine samples. BPD was defined as need for supplemental oxygen at 36 weeks' PMA. Severe ROP was defined as stage 3 or greater. The BWH Institutional Review Board approved the collection of clinical data and urine samples without informed consent. Once subjects were selected, urine samples were removed from the biorepository, marked with identification and sample numbers, and given to the basic science researchers (B.M.L., A.M., D.E.I.) without corresponding clinical data; urine VEGF and protein were measured. De-identified clinical data were submitted by the SCOR group researchers and laboratory data were submitted by the basic science researchers to an independent statistician (L.A.K.) for analysis.

VEGF Measurement

Urine samples were collected using cotton balls placed in the diapers of subjects and stored frozen at –80°C. VEGF was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Human Quantikine ELISA, Minneapolis, Minn., USA) in duplicate samples and averaged values were recorded. Because the immature kidneys of very premature infants allow the passage of excess protein in the urine [13], total urine protein was measured using BD protein assay (Becton Dickinson Co., Franklin Lakes, N.J., USA) to provide a physiologic denominator for urine VEGF protein.

Statistical Analysis

Parametric statistical analyses were performed using logarithmically transformed values because urine VEGF and VEGF/protein data were not normally distributed; results were transformed back to original units for reporting.

Linear random effects models were used to evaluate associations between VEGF or VEGF/protein and gestational age (GA), postnatal age (PNA), and PMA. Models with PNA included both random intercepts and slopes. Associations of VEGF/protein with individual subject characteristics were tested using separate mixed effects models while adjusting for PNA. Potential interactions between each characteristic and PNA were evaluated with the Wald test. Significantly associated characteristics were then considered in multivariate analysis while adjusting for PNA. For reporting, each subject's VEGF/protein measurements were averaged and the medians and quartiles of these values within subgroups were presented. Model-based associations with age were illustrated by plotting the regression lines showing VEGF/protein levels in original units on a multiplicative scale.

We evaluated respiratory support and fraction of inspired oxygen (FiO_2) both as time-fixed (at day 7) and time-varying (on sample day) covariates. To investigate the joint effects of VEGF/protein and day 7 respiratory support on BPD and ROP outcomes, we used logistic regression models using the average of each subject's VEGF/protein levels. We then contrasted odds ratios (ORs) representing the associations between the predictors (VEGF/protein and respiratory support) and outcomes from separate unadjusted models versus from a single multivariable model.

All analyses were performed using SAS 9.2. p values are two-sided and considered significant when $p < 0.05$.

Results

A cohort of 106 subjects was selected (described in table 1) from among 625 eligible SCOR subjects and 257 urine samples were assayed. Cohort subjects were similar to the larger SCOR population in birth weight, GA, gender, and race (data not shown). Each subject contributed

≥2 samples; 45 subjects contributed 3. Most samples were obtained in the first 2 postnatal weeks (147, 94, and 16 samples from weeks 1, 2, and ≥3, respectively).

Changes in Urine VEGF by PNA and Maturity

Unadjusted VEGF did not significantly change with increasing GA, PNA, or PMA (data not shown). VEGF/protein increased with increasing GA at birth (↑8.4% per week GA (95% CI +0.5%, +16.9%, p = 0.04), and then increased more rapidly with increasing PNA (↑72% per week (95% CI +48.3%, +99.5%, p < 0.0001). As PMA reflects both GA and PNA, its effect on VEGF/protein was intermediary (↑19.7% per week PMA (95% CI +11.9%, +28.1%, p < 0.0001). VEGF/protein was used for all subsequent analyses, while controlling for PNA and/or developmental maturity as indicated.

Subject Characteristics and Urine VEGF/Protein

We initially explored associations between multiple characteristics or outcomes and urine VEGF/protein while controlling only for PNA (table 2). Subjects on continuous positive airway pressure (CPAP) or mechanical ventilation (MV) at day 7 had 33.2 and 46% lower VEGF/protein values, respectively, compared with subjects receiving no support (fig. 1a). Urine VEGF/protein was also significantly lower in association with greater respiratory support at the time of the sample, but only during the first postnatal week (interaction test, p = 0.0002; fig. 1b). There was no association between urine VEGF/protein and FiO_2 on day 7 (table 2, p = 0.19) or at the time of sample (p = 0.47).

Many other factors were significantly associated with VEGF/protein when adjusting only for PNA (table 2), but only gender and early sepsis remained associated after adjusting for PNA and respiratory support on day 7 (table 3). We obtained similar results adjusting for PNA and respiratory support on the day of sample.

Association of Urine VEGF with BPD or ROP

Subjects who later developed BPD or ROP had lower urine VEGF/protein values (table 2). Using logistic regression models, we examined the association between VEGF/protein and BPD or ROP while adjusting for respiratory support on day 7. We adjusted only for respiratory support because it was overwhelmingly associated with both VEGF/protein and BPD or ROP, whereas gender and early sepsis were not associated with BPD (both p = 0.56, Fisher's exact test).

A 2-fold decrease in VEGF/protein was associated with a 1.66-fold (95% CI 1.04, 2.65) greater odds of developing BPD (fig. 2a); however, the association was no longer significant once the model was adjusted for respiratory support on day 7 (OR = 1.04, 95% CI 0.58, 1.85). In the analysis of respiratory support, both CPAP and MV were associated with BPD with ORs of 6.72 (95% CI 1.32, 34.3) and 37.6 (95% CI 7.71, 183.2), respectively, compared with no mechanical support. When this model was adjusted for VEGF/protein, the associations between respiratory support and BPD remained virtually unchanged and highly significant. The adjusted analysis yielded similar results if we also adjusted for GA at birth or mean PMA or if we restricted the VEGF/protein values to those collected during the first week of life.

In a similar analysis of VEGF and severe ROP (fig. 2b), MV was compared to the combined reference group of CPAP and no respiratory support, as there were no infants on CPAP on day 7 who developed severe ROP. A 2-fold decrease in VEGF/protein was associated with a 2.82-fold (95% CI 1.45, 5.48) increased odds of developing severe ROP, and MV was associated with an OR of 27.5 (95% CI 3.47, 217.6). Both ORs were attenuated slightly (14–19%) in adjusted analyses but both remained statistically significant. These conclusions were unchanged if we also adjusted for GA at birth or mean PMA or if we restricted the VEGF/protein values to those collected during the first week of life.

Table 1. Demographic characteristics, perinatal factors and outcomes of cohort (n = 106, except where shown)

Demographic characteristics	
GA (mean ± SD), weeks	26.3±1.5
23–26	52 (49%)
27–28	54 (51%)
Birth weight (mean ± SD), g	916.8±235.4
Intrauterine growth restriction	14 (13%)
Male gender	61 (58%)
Non-White race	35 (33%)
Perinatal factors	
Chorioamnionitis	26 (25%)
Pregnancy-induced hypertension	14 (13%)
Magnesium sulfate	42 (40%)
Any antenatal corticosteroid	92 (87%)
Cesarean section	67 (63%)
5-min Apgar score <7	39 (37%)
Outcomes	
Patent ductus arteriosus	65 (61%)
Bronchopulmonary dysplasia	54 (51%)
ROP stage 3–4 or laser (n = 82)	22 (27%)
Intraventricular hemorrhage	32 (30%)
Periventricular leukomalacia	4 (4%)
Necrotizing enterocolitis	15 (14%)

Table 2. Association of characteristics or outcomes with VEGF/protein in unadjusted analyses

Characteristic or outcome	VEGF/protein, pg/mg median (IQR)[a]	% difference[b] (95% CI)[c]	p value[c]
Demographics			
GA, weeks			
<27	11.52 (6.32, 16.51)	−29.8 (−43.4, −13.0)	0.002
≥27	16.12 (9.63, 22.88)	reference	
Birth weight, g			0.99
<750	13.95 (10.15, 18.48)	−2.2 (−26.7, +30.5)	
750–999	11.85 (7.68, 20.68)	−0.7 (−24.1, +29.8)	
≥1,000	12.49 (7.68, 20.20)	reference	
IUGR (<10th percentile)	12.97 (11.02, 20.23)	+7.7 (−23.1, +50.8)	0.66
No IUGR (≥10th percentile)	12.49 (7.56, 19.91)	reference	
Gender			
Male	11.02 (6.70, 16.12)	−34.1 (−46.7, −18.4)	0.0003
Female	18.97 (11.63, 23.97)	reference	
Race			0.21
Other	18.48 (11.82, 20.68)	+30.2 (−4.9, +78.1)	
Black	17.21 (10.15, 21.47)	+14.4 (−15.7, +55.2)	
White	11.95 (6.70, 18.07)	reference	
Perinatal/neonatal factors			
Chorioamnionitis	14.26 (9.11, 24.45)	+27.0 (−1.96, +64.6)	0.07
No chorioamnionitis	12.49 (7.17, 19.21)	reference	
PIH	11.69 (11.02, 15.32)	−14.0 (−38.5, +20.3)	0.37
No PIH	12.90 (7.59, 20.20)	reference	
Prenatal steroids			0.51
Complete	13.80 (8.99, 20.23)	+8.2 (−24.1, +54.3)	
Partial	12.64 (6.58, 19.90)	−6.9 (−36.7, +37.0)	
None	11.58 (5.23, 20.17)	reference	
Magnesium sulfate	10.93 (6.58, 15.35)	−23.4 (−39.0, −3.9)	0.02
No magnesium sulfate	16.27 (9.04, 20.80)	reference	
Cesarean section	12.18 (7.30, 19.86)	−17.5 (−34.6, +4.1)	0.10
Vaginal delivery	14.76 (8.67, 23.97)	reference	
Intubated in DR	11.63 (7.03, 17.29)	−35.9 (−50.4, −17.2)	0.001
Not intubated in DR	20.20 (15.89, 29.04)	reference	
5-min Apgar <7	11.02 (5.89, 17.29)	−27.4 (−42.2, −8.7)	0.007
5-min Apgar ≥7	15.35 (8.99, 21.62)	reference	
Respiratory support on day 7			<0.0001
MV	11.39 (6.05, 15.59)	−46.0 (−58.2, −30.3)	
CPAP	11.75 (8.98, 19.89)	−33.2 (−49.8, −11.0)	
None	20.45 (14.08, 26.46)	reference	
FiO_2 on day 7			
≥0.30	12.39 (7.50, 18.48)	−14.04 (−31.7, +8.25)	0.19
<0.30	12.90 (8.29, 20.80)	reference	
Early sepsis (<72 h)	10.09 (5.71, 15.05)	−34.6 (−47.2, −19.0)	0.0002
No early sepsis	16.23 (11.30, 21.47)	reference	
Late sepsis (≥72 h)	11.39 (7.45, 18.48)	−21.9 (−37.6, −2.2)	0.03
No late sepsis	15.67 (10.55, 22.88)	reference	
Outcomes			
BPD	11.90 (6.05, 17.29)	−24.1 (−39.2, −5.4)	0.02
No BPD	15.51 (8.83, 22.18)	reference	
ROP stage 3–4/laser	6.32 (4.86, 13.00)	−34.3 (−50.3, −13.2)	0.004
No ROP stage 3–4/laser	13.80 (10.57, 20.04)	reference	

DR = Delivery room; IUGR = intrauterine growth restriction; PIH = pregnancy-induced hypertension. [a] Median (IQR) of per-patient values; each per-patient value represents the mean of 2–3 values. [b] Values represent model-based differences in $\log_{10}$(VEGF/protein) from reference, re-expressed as % differences in original units and may differ from % differences of reported median values. [c] Analyzed with separate mixed effects models for each covariate while adjusting for PNA.

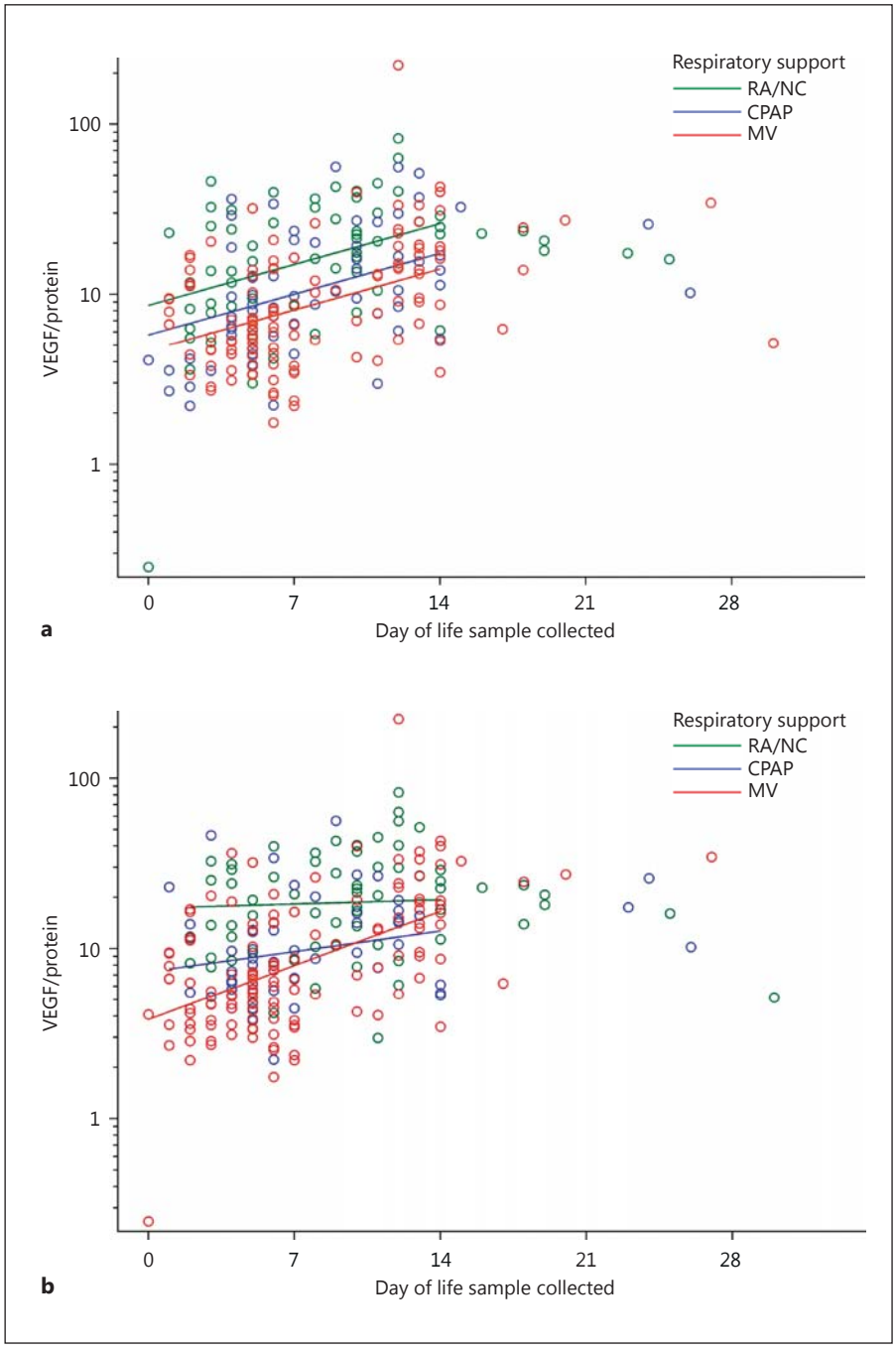

Fig. 1. Association between VEGF/protein and respiratory support at day 7 (**a**) and day of sample (**b**). RA/NC = Room air or nasal cannula. Analysis includes all available data but regression lines are truncated at day 14 as only 6% of samples contributed data beyond 2 weeks of age.

In figure 3, we plotted VEGF/protein values for all subjects using different colors for those who went on to develop BPD or ROP and those who did not. There was substantial overlap between values obtained for affected versus unaffected infants with no apparent cutoff value that distinguished between groups.

Discussion

We found that urine VEGF/protein increased with increasing PNA. Higher levels of respiratory support were associated with lower VEGF/protein, but only in the first week of life, and VEGF/protein levels were lower in males and in subjects with early sepsis. Infants who later devel-

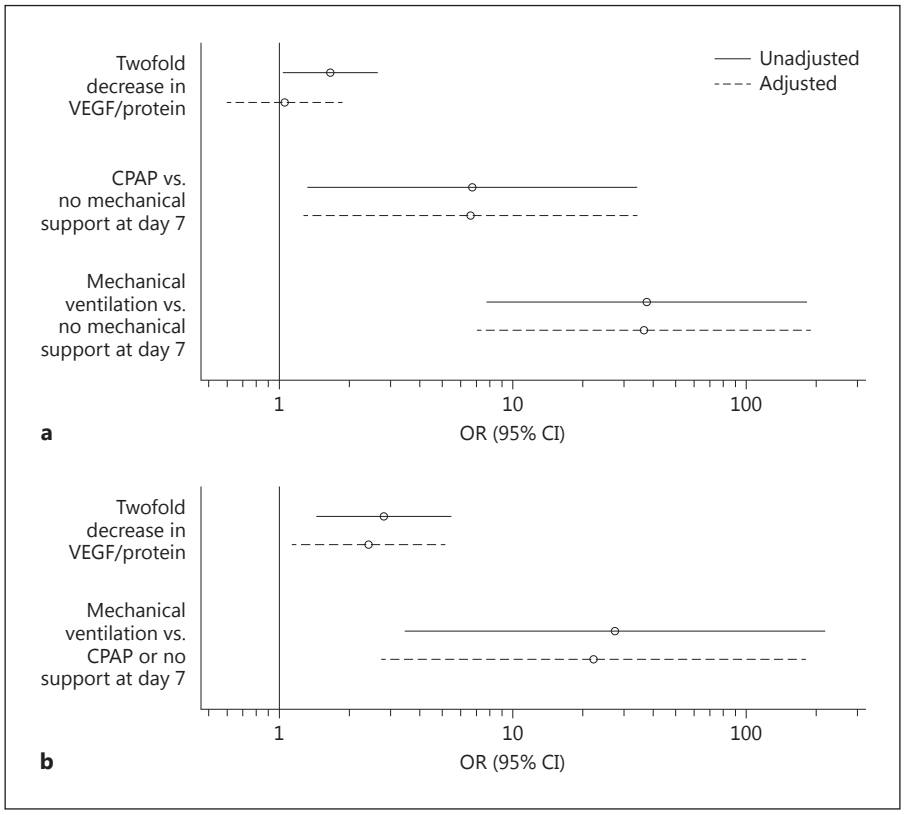

Fig. 2. Logistic regression models for odds of BPD diagnosis (oxygen requirement at 36 weeks' PMA; **a**) and ROP (stage 3–4 or requiring laser photocoagulation; **b**). Unadjusted ORs are from separate logistic models, one for VEGF/protein and one for respiratory support at day 7. Adjusted results are from single logistic model including both predictors.

oped BPD or ROP had lower urine VEGF/protein levels than those who did not. Higher levels of respiratory support were strongly and independently associated with both BPD and ROP. In adjusted analyses, the association between lower urine VEGF/protein and BPD was no longer evident once the analysis was adjusted for respiratory support; however, the association between lower urine VEGF/protein and ROP remained significant.

It is well known that hypoxia induces VEGF expression through hypoxia-inducible factor-1α (HIF-1α) signaling [14], and evidence of an association between urine VEGF/protein and FiO_2 would have been biologically plausible, but was not found. It is possible that exposure of the newborn infant to room air is sufficient to fully suppress the HIF-1α contribution to VEGF expression, thereby preventing further VEGF suppression with FiO_2 >0.21. Or perhaps the VEGF response to FiO_2 is simply overwhelmed by the response to MV.

Regulation of VEGF expression by mechanical forces is not as well studied. Until recently, the huge contribution of mechanical forces in regulating gene expression and organ development had not been fully appreciated, but such forces are very important during lung develop-

Table 3. Association of characteristics or outcomes with VEGF/protein in adjusted analyses

Characteristic or outcome	% difference[a] (95% CI)[b]	p value[b]
Gender		
Male	–28.4 (–41.1, –12.8)	0.001
Female	reference	
Respiratory support on day 7		0.006
MV	–34.3 (–48.9, –15.6)	
CPAP	–24.1 (–42.0, –0.6)	
None	reference	
Early sepsis (<72 h)	–26.5 (–39.8, –10.4)	0.003
No early sepsis	reference	

[a] Values represent model-based differences in $\log_{10}$(VEGF/protein) from reference, re-expressed as % differences in original units and may differ from % differences of reported median values.
[b] Demographic and perinatal/neonatal covariates (not outcomes) were considered in multivariate analysis while adjusting for PNA. Only gender, respiratory support on day 7 and early sepsis remained significant predictors of VEGF/protein.

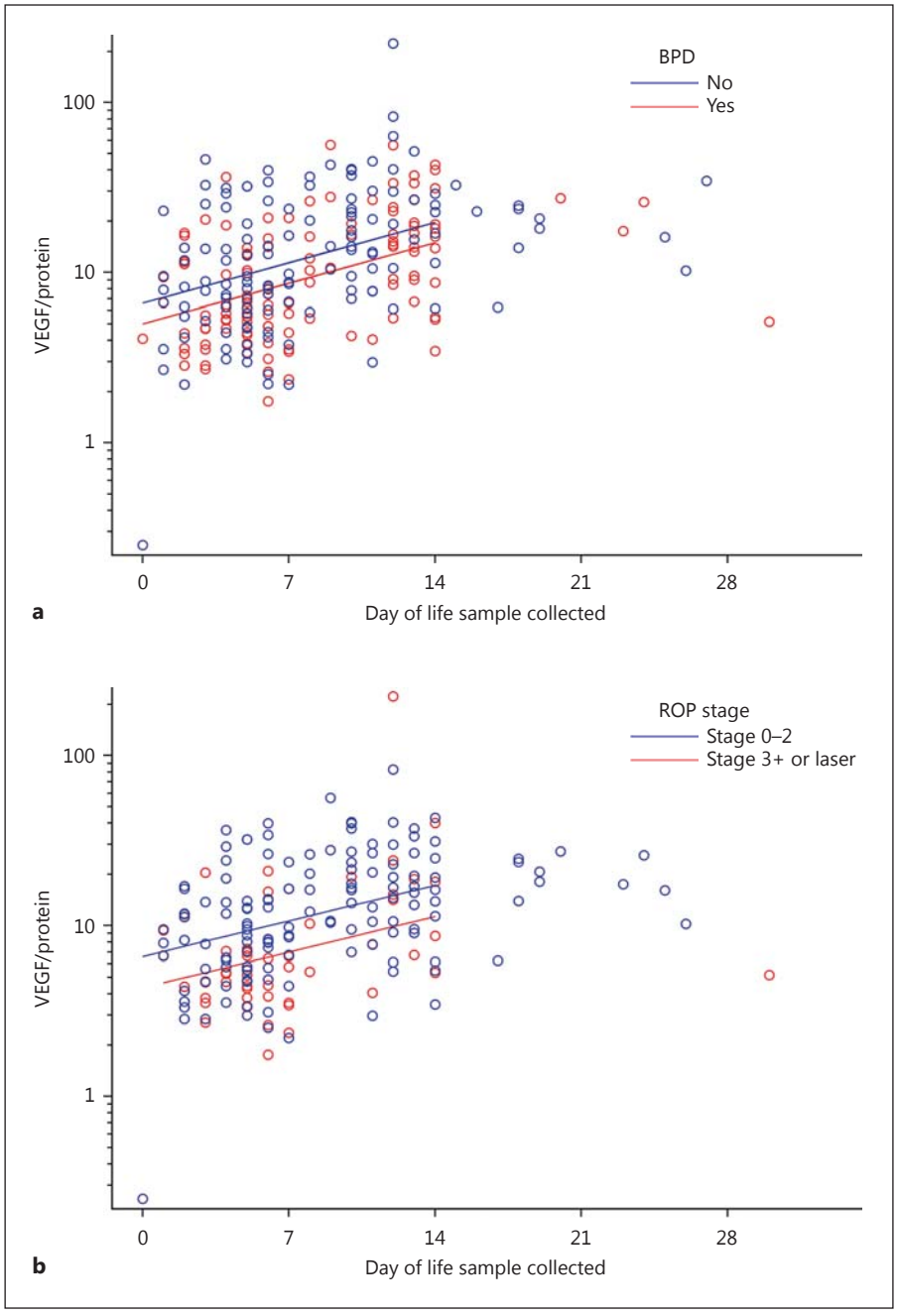

Fig. 3. Association between VEGF/protein and development of BPD (**a**) or ROP (**b**). Analysis includes all available data but regression lines are truncated at day 14 as only 6% of samples contributed data beyond 2 weeks of age.

ment [15–17]. We know that excessive mechanical strain applied to the immature lung with MV alters multiple growth factors and cytokines, disrupts lung development, and leads to BPD [18]. Mechanical strain also regulates lung VEGF expression [19–21], and VEGF is significantly reduced in the lungs of ventilated newborn animals [22]. We found a stepwise decrease in urine VEGF/protein associated with a stepwise increase in respiratory support from none to CPAP to MV. This finding is consistent with preliminary in vitro studies showing progressively less VEGF production with increasing levels of cyclic mechanical strain applied to cultured type II pneumocytes [20].

Study infants who later developed BPD or ROP had lower urine VEGF/protein and were exposed to higher levels of respiratory support. The association between

lower VEGF/protein and BPD was no longer significant once the model was adjusted for respiratory support, but higher levels of respiratory support remained significantly associated with BPD once the model was adjusted for VEGF/protein. This suggests that while respiratory support appears to decrease urine VEGF/protein, respiratory support increases the odds of developing BPD by some mechanism not reflected in urine VEGF/protein. This mechanism could still possibly involve VEGF if the effect is on matrix-associated lung tissue VEGF that is not measured in the urine. Both VEGF/protein and MV were associated with increased odds of developing ROP. Each of these associations was attenuated slightly in adjusted analyses but remained statistically significant. These results suggest that MV and urine VEGF/protein are independently associated with ROP.

The associations we report are remarkable considering the limitations inherent in measuring urine rather than tissue VEGF. There are several VEGF isoforms, but only the diffusible VEGF isoforms ($VEGF^{121}$ and $VEGF^{165}$) can be measured in serum and urine, whereas matrix-bound $VEGF^{189}$ can be measured only in tissue [23]. Serum VEGF is derived from total body VEGF production, not selectively from the lung or eye, and urine VEGF may not closely reflect serum VEGF [24]. Despite these limitations, and despite the very small sample size of this study, our findings lend further support to several prior studies linking low VEGF expression with MV, BPD, and ROP.

In summary, among this population of extremely premature infants, we found that low urine VEGF/protein levels during the first postnatal month were associated with mechanical respiratory support, BPD, and ROP. Larger, population-based studies would be required to determine sensitivity and specificity of urine VEGF in predicting BPD or ROP. Our results suggest that urine VEGF could be an effective biomarker. In the best-case scenario, urine VEGF could be used to identify patients at risk for BPD or ROP early enough to provide some opportunity to intervene. Potential interventions could include reducing exposure to known causative factors (such as oxygen, MV, inflammation) or new medications currently in development that aim to increase VEGF production (for example, inhibitors of prolyl hydroxylase domain-containing proteins that activate hypoxia-inducible factors [6]). Ideally, urine VEGF could be used to study the effect of such interventions in real-time in addition to being used as a predictor of BPD or ROP.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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