Cord Blood Biomarkers of Placental Maternal Vascular Underperfusion Predict Bronchopulmonary Dysplasia-Associated Pulmonary Hypertension

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Objective To assess whether cord blood biomarkers associated with placental maternal vascular underperfusion (MVU) are predictive of bronchopulmonary dysplasia-associated pulmonary hypertension (BPD-PH).

Study design Premature infants enrolled in a longitudinal cohort study were randomly sampled from 4 gestational age strata (n = 190, range 23-36 weeks). Fifteen factors from a human angiogenesis panel were measured in cord blood using multiplex immunoassay. Multivariate linear regression was used to compare biomarker levels according to placental histologic MVU, taking into account acute/chronic inflammation and fetal vascular pathology. Biomarkers associated with MVU were further evaluated in the subgroup of extremely low gestational age infants (gestational age ≤ 28 weeks; n = 48), and measured by enzyme-linked immunoassay in an additional 39 infants to determine associations with BPD (defined using the National Institutes of Health workshop criteria) and PH (identified by echocardiogram at 36 weeks of gestation).

Results Cord blood placental growth factor (PIGF), granulocyte-colony stimulating factor (G-CSF), and vascular endothelial growth factor-A were decreased with MVU ($P < .003$), and decreased with BPD-PH ($P < .05$). The findings were validated for PIGF and G-CSF in 39 additional extremely low gestational age infants. In the combined group (n = 87), PIGF was decreased in infants with BPD-PH (n = 21) versus controls without PH (median 3 pg/mL [IQR 2-7] vs median 15 pg/mL [IQR 6-30], respectively; $P < .001$). G-CSF was similarly decreased with BPD-PH (median, 55 pg/mL [IQR 38-85] vs median 243 pg/mL [IQR 48-1593], respectively; $P = .001$). Receiver operator curve analysis revealed that decreased PIGF and G-CSF were predictive of BPD-PH (area under the curve 0.83 and 0.76, respectively).

Conclusions Cord blood angiogenic factors that are decreased with placental MVU may serve as predictors of BPD-PH. *(J Pediatr 2017;185:33-41).*

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Maternal vascular underperfusion (MVU) is a group of placental histologic lesions seen in births complicated by preeclampsia and intrauterine growth restriction. Findings of MVU include placental vascular bed abnormalities such as fibrinoid necrosis with acute atherosis (FN/AA), and maldevelopment of the villous tree as in distal villous hypoplasia with small terminal villi (DVH/STV).[1] These aberrations in placental morphology are thought to be the result of abnormal placentation, with chronic malperfusion of the chorionic villi by the maternal vascular supply. Chronic fetal hypoxia owing to placental insufficiency, as indicated by the severity and extent of MVU may explain the reported associations between maternal preeclampsia and adverse infant outcomes.[2-4]

BW	Birth weight
BPD	Bronchopulmonary dysplasia
BPD-PH	Bronchopulmonary dysplasia-associated pulmonary hypertension
DVH/STV	Distal villous hypoplasia with small terminal villi
ELGAN	Extremely low gestational age
FN/AA	Fibrinoid necrosis with acute atherosis
FGR	Fetal growth restriction
GA	Gestational age
G-CSF	Granulocyte colony-stimulating factor
MVU	Maternal vascular underperfusion
PH	Pulmonary hypertension
PIGF	Placental growth factor
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor-A

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In a large epidemiologic study, we described the association between placental histologic MVU and the development of pulmonary hypertension (PH) in premature infants with bronchopulmonary dysplasia (BPD).[5] BPD, defined as persistent oxygen dependence at 36 weeks postmenstrual age,[6] affects up to one-third of extremely low gestational age (ELGAN) infants born at <28 weeks of gestation.[7-9] In 25%-40% of infants with BPD, pulmonary vascular disease presenting as PH also persists.[8] This entity, known as BPD-associated PH (BPD-PH) is characterized by more severe cardiopulmonary instability and worse long-term outcomes among survivors.[9,10] BPD-PH is associated with a 4-fold increased risk of death as compared with infants who have BPD without PH,[11,12] yet there are no reliable early biomarkers to distinguish risk.

There is increasing evidence that the pulmonary vascular maldevelopment characteristic of BPD-PH begins even before birth, and that it is mediated by chronic fetal hypoxia.[13] Placental MVU helps to identify infants exposed to such hypoxia. Correlation with systemic markers that can further enhance our understanding of how placental dysfunction impacts fetal lung angiogenesis are needed. Cord blood angiogenic factors that vary with placental histologic MVU are promising indicators, because cord blood is taken directly from the infant's circulation at the time of birth and is a direct correlate of placental function leading up to preterm birth.

The purpose of this study was 2-fold. First, we sought to identify cord blood biomarkers associated with placental MVU, taking into account gestational age (GA) and other important covariates. Our second objective was to determine whether MVU-associated biomarkers are in turn associated with later BPD-PH among the ELGAN infants. Building on our previous report that MVU is associated with the development of PH in infants with BPD,[5] we hypothesized that cord blood biomarkers associated with MVU are predictive of BPD-PH.

Methods

The patient sample was drawn from an ongoing longitudinal cohort study conducted at Prentice Women's Hospital in Chicago. Eligible patients of the parent study are live births ranging from 23 to 36 weeks of gestation. The study was approved by the Institutional Review Board at Northwestern University. Maternal informed consent was obtained before participation of all mothers and their babies. Cord blood is collected at birth, and placentas are sent for gross and histopathologic examination by a perinatal pathologist. Because the multiplex assay platform for the current study accommodated 190 samples in duplicate, we narrowed the sample size to include only singleton patients enrolled between January 2012 and December 2013. Multiple gestation births and infants with congenital anomalies, known genetic syndromes, congenital infections, and metabolic disorders were excluded. Only patients who survived to hospital discharge were included. To control for the influence of GA, we performed stratified sampling of this population (n = 564 infants), partitioning first into 4 strata based on completed weeks gestation at birth: (1) extremely preterm (23-28 weeks, n = 63), (2) very preterm (29-31 weeks, n = 108), (3) moderately preterm (32-34 weeks, n = 272), and (4) mildly preterm (35-36 weeks, n = 121). Simple random sampling was then performed within each strata to obtain 47-48 infants in each of the 4 GA groups, masked to all clinical and placental information. From this sample of 190 births, we identified biomarkers of placental MVU via multiplex immunoassay. After this first analysis, 13 additional ELGAN infants with BPD-PH were identified from the parent study and matched by GA with 13 infants with BPD only and 13 controls without BPD or PH. Cord blood plasma levels from this second cohort of 39 infants were measured via individual enzyme-linked immunoassay, then combined with biomarker data from ELGAN infants from the first cohort.

Maternal and infant data were collected prospectively per the parent study protocol onto standardized abstraction forms that included information on intrapartum management, pregnancy complications, and infant hospital course. GA was assessed with an algorithm based on last menstrual period and ultrasound imaging.[14] Preeclampsia and related complications were defined according to American College of Obstetricians and Gynecologists' criteria.[15] Fetal growth restriction (FGR) was defined as a birth weight (BW) of <10th percentile for GA based on Fenton growth curves.[16] BPD was defined by the National Institutes of Health consensus definition of oxygen requirement at 36 weeks postmenstrual age.[6] All infants who required oxygen at this time point received an echocardiogram evaluation, and PH status was determined according to an algorithm previously published for this cohort.[5,17]

Cord blood was obtained by labor and delivery staff into EDTA tubes, centrifuged for 10 minutes in a tabletop refrigerated centrifuge at 3000 rpm. Plasma was removed from the cell pellet and stored at −80°C until assay. Simultaneous measurement of 15 biomarkers was performed by sandwich immunoassays using Luminex xMAP platform in magnetic bead format. The multiplexed assay beads were obtained from a commercially available kit (Human Angiogenesis/Growth Factor Magnetic Bead Panel 1, HAGP1MAG-12K, EMD Millipore, Massachusetts). The 15 angiogenic proteins were selected from 17 analytes available for this platform based on comprehensive review of the literature for factors directly or indirectly associated with preeclampsia or FGR, that could biologically exert their effects through placental vascular disease: epidermal growth factor, hepatocyte growth factor, and heparin-binding epidermal growth factor[18]; angiopoietin-2, endoglin, vascular endothelial growth factor (VEGF), and placental growth factor (PIGF)[19]; granulocyte colony-stimulating factor (G-CSF) and interleukin-8[20]; endothelin-1[21]; leptin[22]; and fibroblast growth factor-1 and fibroblast growth factor-2.[23] Plasma samples were thawed on ice and prepared in 1:3 dilution. The samples were analyzed on the Luminex platform according to manufacturer's instructions. All samples were run in duplicate with standard curves for each marker and controls on each plate. Intra-assay and interassay coefficients of variation were <15% and <5%, respectively, based on absolute differences in concentrations for each analyte. For further evaluation of the MVU- associated biomarkers measured according to BPD-PH outcomes, individual assays were performed in 1:1 dilution

using commercially available enzyme-linked immunoassay kits according to manufacturer's instructions (R&D Systems, Minneapolis, Minnesota).

Placental tissue samples included sections of membranes, umbilical cord, and ≥2 sections of the placental parenchyma. A comprehensive, standardized histopathology review was performed on the slides by a single perinatal pathologist who was masked to all clinical outcomes. Histologic data were recorded and placentas categorized according to the 4 major histologic domains defined as previously published for this cohort.[5] Briefly, MVU was defined using the criteria by Redline et al.[1] Maternal vasculature of the parietal and basal decidua (vessel changes) included FN/AA, muscularized basal plate arteries, and mural hypertrophy of membrane arteries. Villous hypoxic lesions (villous changes) included infarcts, increased syncytial knots, villous agglutination, increased perivillous fibrin, and DVH/STV. The degree of MVU was graded as severe if ≥1 vascular lesions were present, one or more villous lesions were seen, and the placental weight was <10th percentile for GA.[24] If findings of MVU were present but did not meet all these criteria, a grade of mild MVU was assigned. Placental domains of acute inflammation, chronic inflammation, and fetal vascular pathology were all defined according to our previous publication,[5] and are based on the criteria published by Redline et al.[25,26]

Statistical Analyses

Patient demographics and clinical characteristics were compared using ANOVA for continuous variables and the χ^2 or Fisher exact tests for categorical data. Biomarker levels were reported as median and IQR in picograms per milliliter, and compared using Wilcoxon rank-sum tests. Linear regression models were constructed using log-transformed biomarkers as the main outcome in each model. The 4 histologic domains served as the predictors, using the group without any histologic lesions as the reference. To account for the 15 biomarker comparisons, a Bonferroni-corrected threshold of 0.05/15 = 0.003 was used to determine statistical significance. Biomarkers associated with MVU were further analyzed according to MVU severity, using the group without any MVU as the reference. All models were adjusted for baseline clinical covariates identified by univariate analysis, and models evaluating MVU severity were further adjusted for presence of placental acute inflammation and FGR. In the subsequent analysis of BPD-PH outcomes, multivariate logistic regression models were constructed to evaluate the associations of log-transformed biomarkers on BPD-PH. Receiver operator curve analysis was performed to determine the area under the curve, with adjustment for GA, BW, BPD, and MVU severity. All *P* values were from 2-sided tests and all statistical analyses were performed using STATA/IC software version 13.0 (StataCorp, College Station, Texas).

Results

Table I shows the demographic and clinical characteristics of the study sample according to placental histologic MVU status (none, mild, or severe). The mean GA and BW decreased with increased MVU severity. There were higher rates of cesarean delivery and preeclampsia, and lower rates of spontaneous preterm labor, in the severe MVU group. Of the 48 severe MVU cases, 26 (54%) had FGR but only 19 (40%) were accompanied by preeclampsia. Only 13% of severe MVU cases had

Table I. Demographic, clinical, and placental characteristics of the study sample according to MVU status

	No MVU (n = 90)	Mild MVU (n = 52)	Severe MVU (n = 48)	*P*
Gestational age, wk, mean (SD)	32.5 (3.5)	30.4 (3.6)	31.9 (3.0)	.003
BW (g)	1931.7 (675.1)	1546.0 (589.6)	1424.1 (555.4)	<.001
Maternal age (y)	31.4 (6.4)	31.7 (5.8)	30.4 (6.3)	.53
Male sex, n (%)	49 (54)	30 (58)	28 (58)	.88
Maternal race, n (%)				
Black	17 (19)	12 (23)	17 (35)	
White	35 (39)	21 (40)	17 (35)	.44
Other	38 (42)	19 (37)	14 (29)	
Cesarean delivery	29 (32)	19 (37)	32 (67)	<.001
Spontaneous preterm labor	56 (62)	35 (67)	9 (19)	<.001
Antenatal steroids	40 (44)	28 (54)	22 (46)	.54
Preeclampsia*	13 (14)	5 (10)	26 (54)	<.001
Clinical chorioamnionitis†	5 (6)	5 (10)	0 (0)	.10
Gestational diabetes	8 (9)	9 (17)	10 (21)	.33
Chronic hypertension	4 (4)	1 (2)	5 (10)	.15
Oligohydramnios	1 (1)	0 (0)	1 (2)	.59
FGR‡	10 (11)	6 (12)	26 (54)	<.001
Other placental histologic lesions				
Acute inflammation	41 (46)	25 (48)	6 (13)	<.001
Chronic inflammation	41 (46)	23 (44)	18 (38)	.65
Fetal vascular pathology	18 (20)	12 (23)	16 (38)	.21

Categorical variables were compared using Pearson χ^2 or Fisher exact. Continuous variables were compared using analysis of variance.
*Preeclampsia included eclampsia and HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome.
†Clinical chorioamnionitis was defined based on our previous reports using criteria which included presence of intrapartum fever >38°C with ≥2 of the following signs: elevated maternal white blood cell count, maternal or fetal tachycardia, uterine tenderness, and/or foul-smelling amniotic fluid or vaginal discharge at delivery.
‡FGR was defined as BW for gestational age <10th percentile based on Fenton growth charts.

histologic acute inflammation, which was increased significantly in the non-MVU and mild MVU groups.

The biomarker levels and beta-coefficients from the linear regression models are shown in **Table II**, according to the 4 histologic domains and adjusted for relevant demographics and clinical covariates. Taking into account the 15 individual biomarker comparisons, PIGF, G-CSF, and vascular endothelial growth factor-A (VEGF-A) met the statistical significance threshold ($P < .003$) with MVU. These 3 biomarkers were correlated negatively with MVU (negative beta-coefficient). Conversely, G-CSF and interleukin-8 were correlated positively with acute inflammation ($P < .001$).

Cord blood PIGF, G-CSF, and VEGF-A were further evaluated according to MVU severity (**Table III**). All 3 biomarkers were decreased significantly with severe MVU after adjustment for baseline clinical covariates (model 1). VEGF-A was also decreased with mild MVU. PIGF and VEGF-A remained associated significantly with MVU after further adjustment for placental acute inflammation (model 2) and FGR (model 3). In contrast, G-CSF associations were modified by presence of acute inflammation. Analysis by sublesions revealed that PIGF was decreased with both maternal vessel pathology ($P < .001$ for FN/AA and muscularized basal plate arteries) and all 4 villous sublesions ($P = .001$ for increased perivillous fibrin; $P < .001$ for increased syncytial knots, villous agglutination, and DVH/STV). Decreases in VEGF-A and G-CSF were associated largely with villous changes ($P < .001$ and $P = .009$ for VEGF-A and G-CSF, respectively, with increased syncytial knots; $P = .001$ and $P = .004$ for VEGF-A and G-CSF, respectively, with DVH/STV).

All infants with BPD-PH were born at ≤28 completed weeks of gestation. Six infants with BPD only (4 very preterm and 2 moderately preterm) were not ELGAN. To remove the potential confounding effect of higher GA protecting against PH, we included only ELGAN infants in the remaining analyses. As shown in **Table IV**, all 3 of the biomarkers that were associated with MVU in the larger sample (PIGF, G-CSF, and VEGF-A) were decreased significantly with BPD-PH in multivariate logistic regression models adjusted for GA, BW, BPD severity, and MVU severity. To validate these biomarker findings, we identified 39 additional infants born at ≤28 weeks of gestation in the parent cohort and measured cord blood PIGF, G-CSF, and VEGF-A using individual enzyme-linked immunoassay. The biomarker levels and logistic regression associations for the validation cohort, and for all ELGAN infants combined (n = 87) are shown in **Table IV**. PIGF and G-CSF remained decreased significantly with BPD-PH in the validation and combined groups. VEGF-A was no longer decreased significantly; however, relative VEGF-A to PIGF (calculated as concentration of VEGF/concentration of PIGF for each patient) was increased with BPD-PH. Associations with G-CSF and VEGF-A/PIGF, but not PIGF, were modified upon stratification by GA subgroups (≤26 and >26 completed weeks of gestation). The **Figure** illustrates the distribution of median PIGF and G-CSF according to PH and BPD status. Both markers were decreased in the BPD + PH group when compared with infants who did not have PH at 36 weeks, who had BPD only, or who had neither BPD nor PH. Receiver operator curve analysis for the combined group of 87 ELGAN infants revealed that decreased PIGF (area under the curve 0.83; 95% CI 0.72-0.94) and decreased G-CSF (area under the curve 0.76; 95% CI 0.62-0.90) were predictive of PH.

Discussion

We found that PIGF, G-CSF, and VEGF-A are decreased in cord blood of premature infants exposed to placental MVU, and that these levels also vary according to BPD-PH. Among a diverse panel of cord blood angiogenic markers, PIGF, G-CSF, and VEGF-A were decreased selectively with MVU, as compared with normal placentas and taking into account presence of acute/chronic inflammation and fetal vascular pathology. PIGF and VEGF-A decreased with increasing MVU severity—associations that were not attenuated upon further adjustment for acute inflammation and FGR. In contrast, MVU associations with G-CSF were modified by co-presence of acute inflammation. Further evaluation of the subset of ELGAN infants, with addition of a validation cohort, revealed that PIGF and G-CSF were also decreased with BPD-PH disease. Relative VEGF-A to PIGF (VEGF/PIGF ratio) was increased with BPD-PH. These findings contribute to our growing understanding of the role of delayed fetoplacental angiogenesis in the development of BPD-PH.

MVU is a distinct histologic lesion that is the hallmark of abnormal placentation leading to compromised uteroplacental blood flow during pregnancy. The consequences of this abnormal placentation remain unclear, but hypothesized mechanisms have been implicated in the pathogenesis of preeclampsia and FGR.[1,27,28] The presence of MVU in the placenta may be an indicator of ongoing vascular dysfunction that adversely impacts fetal lung development. We have shown that MVU is prominent in placentas of infants with BPD with PH at 36 weeks.[5] More recently, we conducted immunohistochemical staining of trophoblast vessels and found decreased villous vascularity in placentas of infants with BPD-PH, providing morphometric evidence that early delayed placental angiogenesis mirrors future pulmonary vascular disease.[29] The underlying mechanisms remain unclear. Investigation of cord blood delineates the profile of biochemical factors circulating at the time of birth, and provides insight into the interplay between placental and pulmonary vascular dysfunction in the developing premature infant lung.

There are few previous studies aimed at identifying cord blood biomarkers of MVU. Inclusion of mildly and moderately preterm infants was an essential first step in this study, to determine which of the 15 angiogenic markers were selectively associated with MVU, independent of GA and other important factors such as placental inflammation. Our intent was to cast a wide net with the 15 markers, then objectively narrow our focus to those that were the most highly promising predictors of BPD-PH. This strategy was also necessary to minimize false-positive findings when testing 15 markers in a limited sample of ELGAN infants. Based on our previous study of the association between MVU and BPD-PH,[5] we hypothesized that biomarkers associated with MVU across a wider

Table II. Linear regression associations of 15 cord blood angiogenic factors, according to the presence of MVU and other placental histologic lesions from 190 preterm births

Biomarkers	Placental histologic lesions	Beta ± SE	*P*
Epidermal growth factor	None	REF	–
	MVU	0.07 ± 0.28	.80
	Fetal vascular pathology	−0.20 ± 0.30	.51
	Acute inflammation	−0.11 ± 0.27	.69
	Chronic inflammation	−0.69 ± 0.26	.01
Angiopoietin-2	None	REF	–
	MVU	−0.17 ± 0.07	.01
	Fetal vascular pathology	0.04 ± 0.08	.63
	Acute inflammation	0.09 ± 0.07	.17
	Chronic inflammation	0.05 ± 0.07	.43
G-CSF	None	REF	–
	MVU	−0.69 ± 0.23	.002*
	Fetal vascular pathology	−0.18 ± 0.26	.48
	Acute inflammation	1.90 ± 0.23	<.001*
	Chronic inflammation	−0.13 ± 0.22	.56
Endoglin	None	REF	–
	MVU	0.07 ± 0.07	.23
	Fetal vascular pathology	0.07 ± 0.06	.27
	Acute inflammation	−0.04 ± 0.06	.49
	Chronic inflammation	0.002 ± 0.05	.97
Endothelin-1	None	REF	–
	MVU	−0.01 ± 0.14	.93
	Fetal vascular pathology	−0.18 ± 0.15	.23
	Acute inflammation	−0.25 ± 0.14	.07
	Chronic inflammation	−0.15 ± 0.13	.27
Leptin	None	REF	–
	MVU	0.34 ± 0.19	.07
	Fetal vascular pathology	−0.16 ± 0.21	.45
	Acute inflammation	−0.40 ± 0.19	.03
	Chronic inflammation	−0.11 ± 0.18	.52
Fibroblast growth factor-1	None	REF	–
	MVU	0.08 ± 0.09	.34
	Fetal vascular pathology	−0.21 ± 0.09	.02
	Acute inflammation	−0.12 ± 0.09	.19
	Chronic inflammation	0.03 ± 0.08	.68
IL-8	None	REF	–
	MVU	−0.50 ± 0.18	.01
	Fetal vascular pathology	−0.19 ± 0.19	.33
	Acute inflammation	0.75 ± 0.19	<.001*
	Chronic Inflammation	−0.26 ± 0.17	.12
Hepatocyte growth factor	None	REF	–
	Maternal vascular underperfusion	−0.03 ± 0.11	.76
	Fetal vascular pathology	0.01 ± 0.12	.91
	Acute inflammation	0.22 ± 0.11	.05
	Chronic inflammation	−0.12 ± 0.10	.26
Heparin-binding epidermal growth factor	None	REF	–
	MVU	0.15 ± 0.12	.20
	Fetal vascular pathology	−0.13 ± 0.12	.31
	Acute inflammation	0.04 ± 0.12	.72
	Chronic inflammation	−0.06 ± 0.11	.58
PIGF	None	REF	–
	MVU	−0.80 ± 0.16	<.001*
	Fetal vascular pathology	−0.15 ± 0.17	.38
	Acute inflammation	0.19 ± 0.15	.22
	Chronic inflammation	−0.11 ± 0.15	.45
VEGF-C	None	REF	–
	MVU	−0.01 ± 0.20	.95
	Fetal vascular pathology	−0.32 ± 0.21	.13
	Acute inflammation	0.04 ± 0.20	.85
	Chronic inflammation	−0.40 ± 0.18	.03
VEGF-D	None	REF	–
	Maternal vascular underperfusion	−0.39 ± 0.22	.08
	Fetal vascular pathology	−0.32 ± 0.24	.18
	Acute inflammation	−0.01 ± 0.23	.95
	Chronic inflammation	0.15 ± 0.21	.48
Fibroblast growth factor-2	None	REF	–
	MVU	0.01 ± 0.16	.93
	Fetal vascular pathology	−0.45 ± 0.17	.01
	Acute inflammation	0.05 ± 0.16	.76
	Chronic inflammation	−0.14 ± 0.14	.35
VEGF-A	None	REF	–
	MVU	−0.86 ± 0.22	<.001*
	Fetal vascular pathology	0.22 ± 0.24	.36
	Acute inflammation	0.50 ± 0.21	.02
	Chronic inflammation	−0.29 ± 0.21	.16

IL-8, interleukin-8; *VEGF-C*, vascular endothelial growth factor-C; *VEGF-D*, vascular endothelial growth factor-D.
Multivariate linear regression models were constructed with each log-transformed biomarker as the primary outcome. The 4 major histologic classifications were included in the model, with the group of placentas without any lesions (none) serving as the reference. All models were adjusted for gestational age, BW, sex, maternal race, cesarean delivery, preeclampsia, and spontaneous preterm labor.
*A Bonferroni-corrected *P*-value of <.003 was considered significant.

Table III. Median concentrations (pg/mL) and associations of PIGF, G-CSF, and VEGF-A according to placental MVU severity

Biomarkers	MVU status	Median pg/mL (IQR)	Model 1 Beta (SE)	Model 2 Beta (SE)	Model 3 Beta (SE)
PIGF	None	7 (4-18)	REF	REF	REF
	Mild	6 (3-15)	−0.54 (0.17)*	−0.54 (0.17)*	−0.55 (0.17)*
	Severe	3 (2-4)†	−0.83 (0.20)†	−0.85 (0.21)†	−0.85 (0.21)†
G-CSF	None	127 (53-684)	REF	REF	REF
	Mild	99 (53, 929)	−0.50 (0.28)	−0.46 (0.25)	−0.46 (0.25)
	Severe	59 (38-112)†	−1.08 (0.34)*	−0.60 (0.31)	−0.60 (0.31)
VEGF-A	None	74 (26-527)	REF	REF	REF
	Mild	31 (23-45)†	−1.0 (0.25)†	−0.98 (0.24)†	−0.98 (0.24)†
	Severe	36 (25-91)*	−1.0 (0.30)*	−0.84 (0.30)*	−0.84 (0.30)*

Median biomarker levels were compared with the reference group (none) using Wilcoxon rank-sum nonparametric tests. Beta coefficients were determined using multivariate linear regression models of MVU status on log-transformed biomarkers.
Model 1 adjusted for gestational age (weeks), BW (g), sex, maternal race, caesarean delivery, preeclampsia, and spontaneous preterm labor.
Model 2 included variables adjusted for in model 1 and further adjusted for presence of placental acute inflammation.
Model 3 included the variable adjusted for in model 2 and further adjusted for FGR (BW < 10th percentile).
*$P < .01$.
†$P < .001$.

GA spectrum would be predictive of BPD-PH. Upon identification of the 3 markers from the wider GA pool, we proceeded with the next step, which was to further test these 3 markers in a larger sample of infants born at ≤28 weeks of gestation.

In our study, MVU seemed to have a dichotomous relationship with acute inflammation, present in only 13% of severe MVU and >40% of placentas without MVU (**Table I**). Cord blood G-CSF had a similar distribution among the different histologic domains (**Table II**). In linear regression models, the association between G-CSF and MVU was attenuated by presence of acute inflammation (**Table III**). This association is not unexpected, given the primary role of G-CSF as a proinflammatory cytokine.[30] Decreased levels with severe MVU is a new finding that supports its alternative role in placental vascular development. Furmento et al[31] showed that G-CSF

Table IV. Cord blood levels and logistic regression associations of PIGF, G-CSF, and VEGF-A on BPD-PH

	Biomarker levels in median pg/mL (IQR)			OR (95% CI) for biomarker on BPD + PH	*P*-value
	No BPD or PH	BPD only	BPD + PH		
Original cohort (n = 48)					
n	27	13	8		
PIGF	13 (6, 21)	11 (8, 31)	5 (3, 13)	0.36 (0.14, 0.94)*	.037
G-CSF	438 (99, 2323)	624 (139, 1634)	82 (46, 414)	0.50 (0.26, 0.96)*	.038
VEGF-A	31 (23, 51)	63 (23, 229)	23 (14, 30)	0.17 (0.04, 0.86)*	.032
Validation cohort (n = 39)					
n	13	13	13		
PIGF	18 (9, 30)	16 (7, 68)	2 (2, 6)	0.29 (0.12, 0.70)*	.006
G-CSF	47 (30, 511)	176 (45, 795)	48 (36, 65)	0.39 (0.15, 0.98)*	.046
VEGF-A	51 (38, 177)	53 (40, 106)	68 (48, 171)	1.15 (0.53, 2.46)	.725
Combined (n = 87)					
n	40	26	21		
PIGF					
All ≤ 28 wk	16 (6, 24)	15 (8, 59)	3 (2, 7)	0.34 (0.19, 0.62)*	.001
23-26 wk	20 (9, 23)	30 (8, 105)	4 (2, 10)	0.40 (0.19, 0.87)*	.020
27-28 wk	13 (6, 26)	13 (7, 23)	2 (2, 6)	0.32 (0.11, 0.94)*	.038
G-CSF					
All ≤ 28 wk	179 (47, 1547)	409 (69, 1634)	55 (38, 85)	0.47 (0.29, 0.75)*	.002
23-26 wk	1068 (152, 3179)	789 (246, 3071)	52 (38, 76)	0.42 (0.19, 0.93)*	.033
27-28 wk	127 (46, 740)	92 (50, 468)	55 (36, 88)	0.71 (0.39, 1.31)	.276
VEGF-A					
All ≤ 28 wk	37 (26, 103)	56 (35, 229)	36 (23, 90)	0.82 (0.49, 1.38)	.460
23-26 wk	34 (23, 44)	52 (36, 308)	36 (23, 147)	0.82 (0.42, 1.59)	.557
27-28 wk	42 (30, 103)	61 (24, 167)	39 (23, 68)	0.33 (0.07, 1.60)	.170
VEGF/PIGF					
All ≤ 28 wk	3 (1, 10)	6 (1, 20)	11 (6, 55)	1.51 (1.03, 2.23)*	.037
23-26 wk	3 (1, 8)	5 (1, 21)	11 (6, 55)	1.21 (0.73, 2.0)	.458
27-28 wk	6 (2, 10)	6 (2, 20)	10 (5, 51)	1.28 (0.45, 3.6)	.646

Biomarker levels and ratios reported as median pg/mL (IQR). ORs and *P*-values determined by logistic regression models of the log-transformed biomarkers on BPD-PH, adjusted for gestational age, BW, BPD severity, and MVU severity. Biomarker ratios were calculated as the concentration of VEGF-A divided by concentration of PIGF for each patient. Gestational age strata determined by completed weeks gestation, and includes n = 42 in the $23^{0/7}$-$26^{6/7}$ weeks subgroup and n = 47 in the $27^{0/7}$-$28^{6/7}$ weeks subgroup.
*$P < .05$.

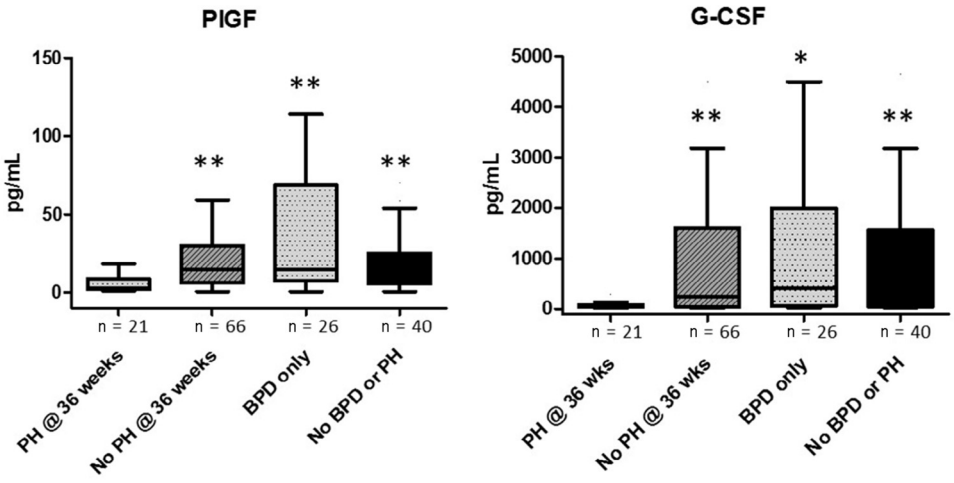

Figure. Cord blood PIGF and G-CSF levels in 87 extremely preterm infants, compared according to BPD and PH status. Infants with BPD-PH disease at 36 weeks corrected age (N = 21) had significantly lower levels as compared with all infants without BPD or PH (N = 66). Levels in infants with BPD-PH were also lower when compared with the subgroups of infants with BPD only but no PH (n = 26), and infants without BPD or PH (n = 40). Median biomarker levels compared using Wilcoxon rank-sum. *$P < .01$ and **$P \leq .001$, versus BPD-PH.

increased VEGF secretion in trophoblast cells, suggesting that G-CSF is involved in the regulation of trophoblast function, implantation, and development of a functional placenta. The distinctions between vascular and inflammatory processes in placenta and cord blood suggest that BPD-PH arises from a pathophysiology that is different from BPD alone. This concept is demonstrated in our previous placental studies,[5,29] and further illustrated in the cord blood biomarker profiles of BPD only versus BPD-PH shown in the **Figure**.

VEGF-A, and perhaps to a greater extent PIGF, are produced by the placental trophoblast throughout pregnancy. Both factors play a role in vasculogenesis and angiogenesis in settings of acute and chronic hypoxia.[32-34] Our findings demonstrate that PIGF and VEGF-A, as they relate to MVU, are not particularly affected by acute inflammation or FGR (**Table III**). Although both factors were significantly decreased with MVU, only PIGF displayed robust association with BPD-PH, independent of GA stratification. VEGF-A levels were not different between infants with or without PH, despite GA differences (**Table IV**). However, relative VEGF-A to PIGF (VEGF/PIGF ratio) was increased in BPD-PH. These findings demonstrate the influence of low circulating PIGF at birth in the development of PH disease. Most notable were the significantly decreased PIGF levels with placental vessel changes FN/AA and muscularized basal plate arteries, and villous changes such as DVH/STV. These specific MVU sublesions were highly associated with development of BPD-PH in our previous report involving this cohort.[5]

The role of PIGF in fetal lung development is not well-understood. Low levels circulating at birth may represent a primary defect in production by the dysfunctional placenta, upstream regulators, or competition with VEGF-A for the receptor antagonist sFlt-1.[32] Consistent with our findings, Procianoy et al[35] reported an increased ratio of VEGF/PIGF in postnatal blood samples (within 72 hours) from infants with BPD, suggesting that the relative VEGF-A to PIGF concentrations observed at the time of birth persist in the immediate postnatal period. Further studies are needed to assess how PIGF and VEGF-A levels vary over time, and whether the imbalance in angiogenic factors contributes to the development of BPD only, BPD with PH, or both. Yang et al[36] found that cord blood PIGF was increased with BPD in a cohort of preterm infants of a much wider GA range (<35 weeks of gestation). In our study, we removed confounding owing to greater GA by restricting our observations to ≤28 weeks of gestation, which may account for differences in results. In fact, when including a similar cutoff to Yang (<35 weeks of gestation) in our sample, we found that PIGF was indeed higher among infants with BPD as compared with controls without BPD (median 13.4 pg/mL vs 6.5 pg/mL; $P = .02$), but only when infants with BPD-PH were excluded. These findings emphasize the importance of taking into account degree of prematurity and presence of PH disease when identifying biomarkers associated with BPD.

Another new finding was the close correlation between PIGF and G-CSF with BPD-PH. G-CSF is a glycoprotein that stimulates bone marrow to produce granulocytes and stem cells. Production by the placenta is not well-described, but it is likely that G-CSF serves as a mediator of PIGF expression by regulating release of monocyte progenitors through which PIGF induces angiogenesis.[37] For example, G-CSF and PIGF have been shown to work synergistically in animal models to mobilize primitive blood progenitor cells.[38] These undifferentiated cells might include endothelial progenitors and fetal monocytes that circulate during pregnancy and at birth, contributing to the pathophysiology of BPD-PH.[39,40] Therefore, another intriguing hypothesis for the link between placental MVU and

BPD-PH is that they share common circulating progenitor cells during fetal development, which predispose to parallel abnormal placental and pulmonary vascular development. Further studies are needed to determine the complex interplay between PIGF, G-CSF, and MVU in the pathogenesis of BPD-PH.

Limitations of this study include the small sample size, single-center cohort, and limited number of markers measured for what potentially remains to be discovered. For example, further studies are needed to determine the role of receptor antagonists such as sFlt-1 that compete for PIGF and VEGF, and upstream regulators such as HIF-1alpha that further determine the angiogenic response to hypoxia. The biomarker associations do not delineate causal mechanisms, especially when evaluating placental and cord blood markers with long-standing PH disease at 36 weeks of gestation. These pathways warrant further investigation using serial echocardiograms and biomarker measurements to track the evolution of BPD-PH. A larger sample of ELGAN infants is needed to further investigate the complex interactions between PIGF, G-CSF, and VEGF-A, and to determine whether other placental markers in combination with cord blood enhances positive predictive value in the clinical setting. In cases where placental tissue is not available readily, cord blood biomarkers may serve as valuable proxies for assessing MVU-mediated PH disease in premature infants.

In conclusion, cord blood biomarkers of placental MVU are predictive of BPD-PH. Our findings support the growing evidence that fetal exposures may have lasting impact on long-term cardiopulmonary health. These markers may serve as indicators of the fetal pathophysiology that gives rise to a distinct vascular phenotype of BPD with PH disease, and may inform the development of novel approaches to individualize the management of multifactorial BPD. ■

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