

BASIC SCIENCE ARTICLE



Placental proteins with predicted roles in fetal development decrease in premature infants

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BACKGROUND: Emerging evidence from animal experiments indicate that factors secreted by the placenta are critical for normal fetal organ development. Our objective was to characterize the umbilical vein and artery proteome in preterm infants and identify proteins that decrease in the neonatal circulation following delivery.

METHODS: Cord blood at delivery and neonatal blood at 48–72 h of life was collected in 25 preterm infants. Plasma protein abundance was determined using the SomaLogic platform.

RESULTS: When comparing protein levels of umbilical venous to arterial cord blood, 434 proteins were significantly higher indicating placental secretion into the fetal circulation. Moreover, when comparing neonatal blood to umbilical vein levels, 142 proteins were significantly lower. These proteins included Endoplasmic reticulum resident protein 29, CD59, Fibroblast growth factor 2 and Dynactin subunit 2, which are involved in brain development and prevention of brain damage as well as Fibroblast growth factor 1 which prevents lung fibrosis.

CONCLUSIONS: The late second trimester human placenta secretes proteins into the fetal circulation which decrease following delivery. Many of these proteins are predicted to be important in the development of fetal organs. Further studies are needed to directly link placental proteins to organ development and poor outcomes in preterm infants.

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IMPACT:

- Prematurity remains a leading cause of morbidity and mortality requiring the development of novel treatments. Emerging evidence from animal studies suggest that factors secreted from the placenta may be critical in the development of the fetus.
- We report that the preterm human placenta secretes an array of proteins into the fetal circulation. Some of these proteins are predicted to be involved in the development of the brain and the lung.
- When born prematurely, infants are deprived of these placental proteins, which may contribute to their poor outcomes.

INTRODUCTION

Each year 12–18 million preterm infants (before 37 weeks of gestation) are born worldwide,^{1–3} representing approximately 10–11% of all live births.^{1–4} Despite improvements in clinical management within the field of perinatal medicine, such as antenatal steroid administration and surfactant replacement, prematurity remains a leading cause of infant morbidity and mortality² with a survival rate of 45% at 23 weeks and 82% at 25 weeks of gestation.⁵ Moreover, those that survive are at increased risk for developing major morbidities including early or late sepsis, necrotizing enterocolitis, chronic lung disease, severe intraventricular hemorrhage (IVH), periventricular leukomalacia (PVL), or severe retinopathy of prematurity (ROP).⁶ Importantly, with each additional co-morbidity, specifically brain injury, ROP, or lung injury, there is an independent association with poor outcome later in life defined as death or disability.^{7–9} Thus, given the large burden of disease in preterm infants, there is an urgent need for the development of novel intervention strategies and treatments.

One of the most fundamental differences between fetal and postnatal life is the instantaneous discontinuation of the umbilical circulation at delivery. In addition to providing nutrients and oxygen to the growing fetus, the human placenta secretes >100 proteins into the maternal circulation, which are involved in the maternal metabolic and cardiovascular adaptations to pregnancy.^{10,11} Moreover, emerging evidence from animal experiments indicate that factors secreted by the placenta into the fetal circulation are critical for normal organ development of the fetus. For example, the development of the fetal forebrain in mice is critically dependent on placental serotonin synthesis,¹² and altered placental serotonin secretion causes abnormal neurodevelopment in adult offspring.^{12,13} Recently, we identified 341 proteins which were significantly higher in the umbilical vein compared to the umbilical artery in normal term infants, representing proteins secreted by the human placenta into the fetal circulation at the end of gestation.¹⁴ However, it is currently unknown if these or other proteins are secreted by the placenta

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into the fetal circulation earlier in gestation and if the preterm neonate can produce these proteins once separated from the placental circulation.

In the present study, we characterized the extremely and very preterm infant proteome as well as identified proteins with significantly decreased circulating concentrations in neonatal as compared to umbilical vein blood.

METHODS

Study subjects

Pregnant women aged 18–45 years who were admitted to labor and delivery at 23 to 31 weeks of gestation due to suspected preterm labor, premature rupture of membranes, pre-eclampsia, and/or other maternal complications were recruited from July 2018 to March 2020 following written informed consent. The Institutional Review Board at University Hospital Colorado and Children's Hospital Colorado approved the study (IRB #18-0637). Exclusion criteria included delivery after 32 and 0/7 weeks of gestation, infants with a congenital cardiac disease (did not include patent ductus arteriosus, patent foramen ovale, or atrial heart defect), malformations or syndromes with unknown genetic defect, genetic abnormality including but not limited to Trisomy 13, 18, and 21, prenatal ultrasound with findings of absent or reverse end-diastolic flow in the umbilical artery, congenital infections including HIV, Hepatitis B or C, or CMV, metabolic disorder, maternal recreational drug use except for alcohol use, infants requiring a blood transfusion prior to the neonatal blood sample, and/or any neonate who was deemed by the medical staff as too ill. These exclusions were chosen given they may have impacted placental/neonatal development, placenta blood flow, or altered the neonatal plasma proteome.

Collection of blood samples and data collection

Within 30 minutes following delivery, umbilical vein and arterial blood was collected from the placenta after clamping of the cord and processed immediately by centrifugation at 2200×g for 15 min at 4°C. Plasma was then collected and subsequently aliquoted and stored at –80°C for later batch analysis. If the neonate met criteria, at 48–72 h of life, 0.6 mL of neonatal blood was obtained, and plasma was collected as above. All samples were de-identified for storage and after collection of relevant data from the electronic medical record. Data on the current pregnancy, mother, and infant at birth and at the time of discharge or death of the patient was recorded in a REDCAP electronic database hosted at Colorado Clinical & Translational Sciences Institute (CCTSI).^{15,16}

SOMOLogic proteomics

Quantification of proteins was performed on paired blood samples (umbilical vein versus umbilical artery and umbilical vein versus neonatal blood) using the SomaLogic platform (Boulder, CO) as previously described.^{17,18} Briefly, each plasma sample was incubated with modified aptamers, which are chemically modified nucleotides that bind to each of the proteins being measured, to generate modified aptamer-protein complexes which are highly specific and stable. Following a series of washes, which disrupt all non-specific interactions, the SOMAmers were placed in a denaturing buffer, releasing them from their target-protein complexes. The released SOMAmers were then hybridized to their complementary sequences on a microarray chip and quantified by fluorescence using an Agilent hybridization array (Agilent Technologies). The resulting signal intensity is directly correlated to the protein levels of the original sample.^{17,18}

Intra-assay variability was estimated and found to be <3% for 50% of the proteins and <8% for 95% of proteins. Inter-assay CVs are not available for our study. However rigorous estimates of inter-assay variability for this particular SOMAscan Assay (1.3 K) were recently reported to be as low as <7% for 50% of the proteins and <15% for 95% of proteins, when using Hyb.Cal normalized data.¹⁹

RNA isolation and qRT-PCR analysis

To assess gene expression of placental proteins, placentas were collected within 60 min of delivery. Three samples of villous tissue (~0.5 cm) were dissected from the placenta and rinsed in ice cold physiologic saline and placed in RNAlater (Qiagen, Venlo, Netherlands) at 4°C for 24–48 h. The RNAlater was removed and samples were stored at –80°C until batch

analysis. RNA was extracted from the villous tissue using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). We selected 6 candidate proteins with the largest concentration differences between the umbilical venous and neonatal circulation with predicted roles in neurogenesis and lung development (Table 5) which included CD59, Dynactin subunit 2, Endoplasmic reticulum resident protein 29 (ERp29), Ephrin-B1, Fibroblast growth factor 1 (FGF1), and Fibroblast growth factor 2 (FGF2). qRT-PCR was carried out using primers from ThermoFisher Scientific, Waltham, MA and RNA analysis was performed as previously described.²⁰ beta actin and 18 S (ThermoFisher Scientific, Waltham, MA) were used as housekeeping genes.

Statistics

Differences in relative abundance of proteins in paired umbilical venous blood and neonatal plasma and umbilical venous and artery blood were compared using paired t-tests. Additionally, we used two-sample t-tests to compare differences in umbilical venous plasma in newborns for the following clinical characteristics: prematurity, placental pathology for chorioamnionitis, delivery method, premature rupture of membranes, pre-eclampsia, intubation at delivery, and sex. The Benjamini-Hochberg procedure was performed to control for false discovery rate given multiple comparisons. Data were transformed using log base 2 and differences are reported as % change with confidence intervals. Significance was set at 0.05. R version 4.0.2 software (R Foundation for Statistical Computing, Vienna, Austria, <http://www.R-project.org/>) was used for analysis.

Bioinformatics and text mining methods

To screen for the most significant pathways of the proteins with the highest or lowest abundance in neonatal blood when compared to the umbilical vein, the Reactome, a pathway database that is utilized to link human proteins by their known molecular functions or to determine novel functional relationships,^{21,22} was utilized. The UNIPRO identifiers for the proteins of interest were introduced into the database and the pathway analysis report was obtained. In addition, we also tested for enrichment of proteins associated with relevant keywords in MEDLINE abstracts such as the development of the brain, lung, intestine, cardiovascular system, retina, neurogenesis, and angiogenesis using text mining methods.²³

RESULTS

Clinical characteristics

As shown in Fig. 1, of the 58 consented patients, 24 patients were screen failures prior to delivery leaving 34 eligible mothers and 35 eligible infants at delivery. This included 4 twin deliveries, with only one set having both twins initially eligible. Ten additional study subjects were excluded during or after delivery due to marked hemolysis in the collected blood sample, inability to collect cord blood secondary to placental abruption or an avulsed cord, or severe anemia in the infant resulting in a blood transfusion, which included one infant where both twins were initially eligible. As a result, 25 mother/infant pairs completed the study.

Baseline and demographic characteristics of the 25 mothers and infants at birth are presented in Table 1. Median maternal age was 33 years with a range of 23 to 39 years. The majority of mothers were non-Hispanic (68%) and white (72%). Preterm premature rupture of membranes complicated 48% of pregnancies, whereas 32% of women in the cohort had pre-eclampsia. Basic infant characteristics at birth included a male predominance (60% males) and a median gestational age at delivery of 30 weeks (range of 24 to 32 weeks). Within the first 6 h of life, 44% of infants required intubation and 52% of infants received antibiotics.

Infant outcomes at discharge are summarized in Table 2. Moderate or severe chronic lung disease was diagnosed in 21.7% of infants, grade III/IV IVH in 13%, stage III-V ROP in 16.7%, and medical and surgical necrotizing enterocolitis in 4% of neonates, respectively.

Proteome of the very and extremely preterm infant

Of the 1,317 proteins on the SomaLogic proteomic platform, 434 proteins were significantly higher in the umbilical vein as

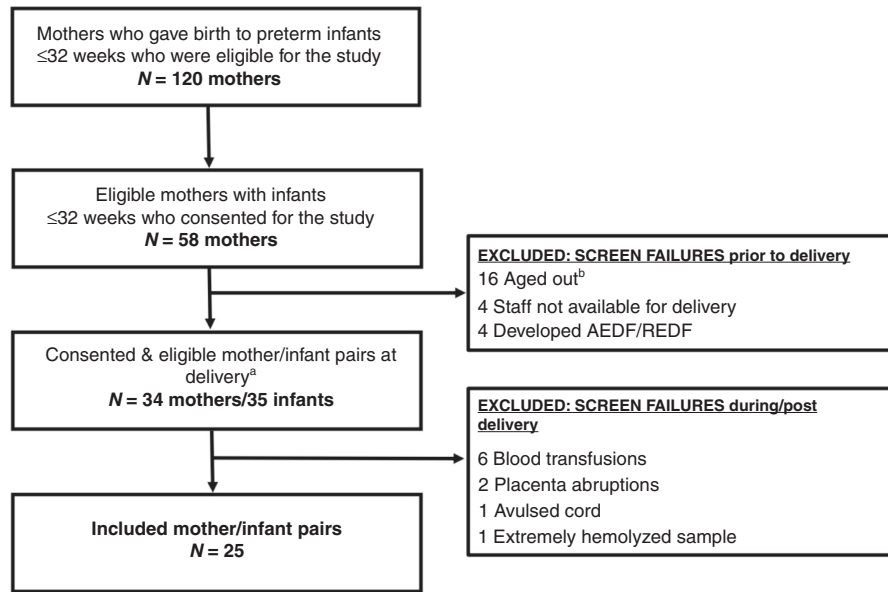


Fig. 1 Flow diagram of patient population. ^a Four sets of twins were included in the study; 3 sets only had 1 twin eligible at the start of the study. One set initially had both twins eligible, however 1 infant required a blood transfusion secondary to extreme anemia and became a screen failure. ^b Aged out indicated those mothers who stayed pregnant past 32 weeks 0 days gestation and therefore were no longer eligible for the study. *AEDF/REDF*, Absent end-diastolic flow/ reverse end-diastolic flow.

compared to the umbilical artery representing secretion or release by the placenta into the fetal circulation with 139 proteins having a $\geq 100\%$ and 18 proteins with a $\geq 500\%$ higher abundance in the umbilical vein (Table 3a and Supplemental Table 1). There were also 302 proteins significantly lower in the umbilical vein as compared to the artery suggesting uptake by the placenta with 84 proteins having a $\geq 50\%$ and 15 proteins having a $\geq 65\%$ lower abundance in the umbilical vein (Table 3b and Supplemental Table 2). In infant blood collected 48–72 h after delivery, the abundance of 142 proteins were significantly lower when compared to the umbilical vein with 25 proteins having a $\geq 50\%$ decrease in neonatal blood when compared to umbilical venous blood (Tables 4a, 5, and Supplemental Table 3). In contrast, 126 proteins were significantly higher in infant blood 48–72 h post-delivery as compared to the umbilical vein with 24 proteins having a $\geq 100\%$ increase in neonatal blood (Table 4b and Supplemental Table 4). Of the 142 proteins significantly decreased in neonatal blood 48–72 h of life, 63 proteins were also secreted or released by the placenta (Supplemental Table 5). When comparing the results of the current study with our previous report on secreted proteins in the term human placenta,¹⁴ we found that 265 of the 434 proteins secreted by the preterm placenta were also released into the fetal circulation by the term placenta (Supplemental Table 6).

Sub-analysis of specific prenatal and post-natal characteristics can be found in Supplemental Table 7. The comparison of the proteomic profiles of venous cord blood from extremely and very preterm infants were similar apart from 3 proteins which were significantly higher in the very preterm infants' venous cord blood when compared to the extremely preterm infants when adjusted for multiple comparisons and 3 proteins which were significantly lower. Additionally, in infants born to mothers with placental pathology positive for chorioamnionitis, 7 proteins were significantly higher in comparison to venous cord blood from infants without chorioamnionitis. Comparing venous cord blood from infants born with the following characteristics: delivery method, premature rupture of membranes, pre-eclampsia, intubation at delivery, and sex, the proteomic profiles between each of the above groups were similar following adjustment for multiples comparisons (Supplemental Table 7).

Bioinformatics

Using the Reactome to determine the biological networks for the 142 proteins that were lower in neonatal blood as compared to the umbilical vein, the immune system, intracellular signaling by second messengers, axon guidance, and nervous system development were some of the most significant pathways (Supplemental Table 8a and ref. ²⁴). Similarly, for the 126 proteins that were increased in the neonate, the immune system, complement cascade, extracellular matrix organization, platelet degranulation, and clotting cascade were identified (Supplemental Table 8b and ref. ²⁵). Using text mining methods on the 142 proteins with significantly lower levels in neonatal than in umbilical vein blood, we found that many of these proteins are associated with the development and/or function of the brain, lung, intestine, cardiovascular system, or retina. A selected number of these proteins, including CD59, Dynactin subunit 2, Ephrin-B1, ERp29, FGF1, and FGF2, and the associated developmental related literature are presented in Table 5.

Messenger RNA analysis

CD59, Dynactin subunit 2, Ephrin-B1, ERp29, FGF1, and FGF2 were found to be expressed on the mRNA level in preterm placentas, indicating they are produced in the placenta. We found similar mRNA expression levels in very and extremely preterm placentas (Fig. 2).

DISCUSSION

We report for the first time the umbilical and post-delivery proteome of extremely and very premature infants using the SOMAscan aptamer-based platform. Our data supports the concept that the late second trimester human placenta secretes proteins into the fetal circulation and using bioinformatics we predict that many of these proteins are involved in the development of fetal organs, including the brain and lung. We speculate that when born prematurely, infants are deprived of these developmentally active proteins, which may contribute to their poor outcomes.

We previously reported that 341 proteins are secreted by the human placenta into the fetal circulation at term.¹⁴ Interestingly,

Table 1. Characteristics of mothers and infants at birth.

Maternal characteristics	
Maternal age, years, median (range)	33 (23–39)
Hispanic ethnicity, <i>n</i> (%)	8 (32%)
Race:	
White, <i>n</i> (%)	18 (72%)
African American, <i>n</i> (%)	2 (8%)
Asian, <i>n</i> (%)	1 (4%)
Unknown, <i>n</i> (%)	4 (16%)
Cesarean Delivery, <i>n</i> (%)	17 (68%)
Single Gestation, <i>n</i> (%)	21 (84%)
Pre-eclampsia, <i>n</i> (%)	8 (32%)
PPROM, <i>n</i> (%)	12 (48%)
Hypertension, <i>n</i> (%)	5 (20%)
Diabetes, <i>n</i> (%)	3 (12%)
Alcohol use, <i>n</i> (%)	2 (8%)
Betamethasone:	
0–1 dose, <i>n</i> (%)	0 (0%)
2 doses, <i>n</i> (%)	12 (48%)
3 doses, <i>n</i> (%)	3 (12%)
4 doses, <i>n</i> (%)	10 (40%)
Chorioamnionitis:	
Clinically + Placental pathology, <i>n</i> (%)	2 (8%)
Placental pathology only, <i>n</i> (%)	13 (52%)
Neonatal Characteristics at Birth	
Gestational age, weeks [median (range)]	30 (24–32)
Male gender, <i>n</i> (%)	15 (60%)
Growth parameters at delivery	
Birth Weight, grams [median (IQR)]	1355 (1040–1700)
Length, cm [median (IQR)]	39 (34–41)
Head circumference, cm [median (IQR)]	28 (25–30)
Respiratory support at delivery	
HFOV, <i>n</i> (%)	2 (8%)
Conventional ventilation, <i>n</i> (%)	9 (36%)
Non-invasive support, <i>n</i> (%)	14 (56%)
Additional support	
Surfactant, <i>n</i> (%)	10 (40%)
Blood pressor support, <i>n</i> (%)	3 (12%)
Antibiotics, <i>n</i> (%)	13 (52%)

N = 25 mothers/infants; *IQR* Interquartile range, *PPROM* Preterm premature rupture of membranes, *HFOV* High frequency oscillatory ventilation.

of these 341 proteins, 265 were also found to be secreted by the preterm placenta into the fetal circulation in the current study, suggesting that the human placental secretome into the fetal circulation does not change markedly in the second half of pregnancy. Suski and colleagues identified 245 proteins at delivery in umbilical cord blood from infants ≤ 30 weeks of gestation as well as at term using liquid chromatography-mass spectrometry with 58–65 of those proteins being significantly higher in concentration in infants ≤ 30 weeks when compared to term.²⁶ When comparing late preterm infants (32 to 36 weeks of gestation) with and without respiratory distress syndrome, Hu and colleagues found that umbilical cord blood from infants with respiratory disease had 139 upregulated and 112 downregulated peptides, many of which were involved in processes of respiratory

Table 2. Clinical characteristics of neonates at discharge.

Neonatal characteristics at discharge	
Living, <i>n/N</i> (%)	24/25 (96%)
DOL at discharge, median (IQR) ^a	67 (47–85)
Postmenstrual age, median (IQR) ^a	39 (38–41)
Chronic Lung Disease ^a	
None, <i>n/N</i> (%)	1/23 (4.3%)
Mild, <i>n/N</i> (%)	17/23 (73.9%)
Moderate, <i>n/N</i> (%)	1/23 (4.3%)
Severe, <i>n/N</i> (%)	4/23 (17.4%)
IVH Grade III/VI, <i>n/N</i> (%) ^b	3/23 (13.0%)
ROP Grade III–V, <i>n/N</i> (%) ^c	3/18 (16.7%)
ROP Any Intervention, <i>n/N</i> (%)	0/18 (0%)
Medical NEC, <i>n/N</i> (%)	1/25 (4%)
Surgical NEC, <i>n/N</i> (%)	1/25 (4%)
Medical intervention for PDA, <i>n/N</i> (%)	3/25 (12%)
PDA Coil, <i>n/N</i> (%)	1/25 (4%)
PDA ligation, <i>n/N</i> (%)	0/25 (0%)

^aTwo patients were transferred to a level 2 NICU and therefore their discharge information was incomplete.

^bOne patient declined the evaluation and one patient transferred prior to the second head ultrasound.

^cSix patients did not qualify for ROP exams given they were >30 weeks and birthweight >1500 g and one patient unknown since transferred prior to exam.

DOL day of life, *IQR* interquartile range, *IVH* intraventricular hemorrhage, *ROP* retinopathy of prematurity, *NEC* necrotizing enterocolitis, *PDA* patent ductus arteriosus.

failure, atelectasis, and maturation of endothelial cells.²⁷ Here we sought to further analyze the preterm umbilical cord plasma proteome as well as determine to what extent the circulating concentrations of these proteins changed in the circulation of preterm infants following delivery. The analysis consisted of approximately 1,300 known proteins in the SOMAmer (Slow Off-rate Modified Aptamer) panel. With $\sim 20,000$ protein coding genes and at least 17,000 identified nonmodified human proteins,²⁸ it can be estimated that the SOMALogic platform used in the current study, represents a 7–8% coverage of the total proteome. Although this platform included only a fraction of all proteins, representing a limitation of our study, the proteins on this platform have been preselected with a focus on secreted proteins.¹⁸ And since our study focused on identifying proteins secreted by the human preterm placenta into the fetal circulation, this provided the rationale for using this proteomic approach in our study. Additionally, the human plasma proteome is highly complex^{29,30} with multiple posttranslational modifications and a profound dynamic range in the concentrations of different plasma proteins,³¹ which constitute a major analytical challenge using traditional LC/MS-based proteomics approaches to identify proteins. For example, in order to measure proteins that are present in plasma in lower concentrations it is necessary to first remove high-abundance proteins, such as albumin, which may result in the concomitant removal of low-abundance proteins, such as cytokines, peptide hormones and lipoproteins. The SOMALogic proteomic platform, however, allows for measurement of proteins in small volumes of serum or plasma with very high sensitivity and dynamic range thereby circumventing some of the major problems with traditional proteomics in plasma.¹⁸ As a consequence, the SOMALogic platform is now widely used in high impact human proteomics studies,^{32–37} including studies in children.^{38–40}

Table 3. Proteins with the largest umbilical vein-to-artery differences in relative abundance.

Target Full Name	UniProt	% Difference (CI)	P-value	Adjusted P-value
A. Proteins with Higher Relative Abundance in the Umbilical Vein				
Netrin receptor UNC5D	Q6UXZ4	2004 (407, 8637)	0.0006	0.0111
Ephrin-B2	P52799	1837 (335, 8531)	0.0011	0.0111
SLIT and NTRK-like protein 5	O94991	1534 (286, 6813)	0.0013	0.0111
Osteopontin	P10451	1375 (262, 5909)	0.0014	0.0111
Leucine-rich repeat transmembrane protein FLRT3	Q9NZU0	844 (182, 3058)	0.0018	0.0111
Collectin-12	Q5KU26	787 (202, 2504)	0.001	0.0111
Properdin	P27918	692 (164, 2280)	0.0016	0.0111
Cystatin-D	P28325	662 (195, 1872)	0.0006	0.0111
Nucleoside diphosphate kinase B	P22392	663 (217, 1735)	0.0003	0.0111
Thyroid Stimulating Hormone	P01215.P01222	629 (174, 1837)	0.0009	0.0111
Growth factor receptor-bound protein 2	P62993	562 (169, 1529)	0.0007	0.0111
Brother of CDO	Q9BWW1	560 (153, 1625)	0.0012	0.0111
Granulocyte colony-stimulating factor	P09919	551 (147, 1616)	0.0014	0.0111
Semaphorin-6A	Q9H2E6	531 (149, 1500)	0.0011	0.0111
Importin subunit beta-1	Q14974	528 (142, 1528)	0.0014	0.0111
Adapter molecule crk	P46108	520 (144, 1475)	0.0012	0.0111
Inorganic pyrophosphatase	Q15181	521 (139, 1508)	0.0014	0.0111
Xaa-Pro aminopeptidase 1	Q9NQW7	513 (168, 1304)	0.0005	0.0111
B. Proteins with Lower Relative Abundance in the Umbilical Vein				
GTP-binding nuclear protein Ran	P62826	-93 (-98, -76)	0.0006	0.0111
40 S ribosomal protein S3	P23396	-83 (-94, -50)	0.0041	0.0128
6-phosphogluconate dehydrogenase, decarboxylating	P52209	-76 (-89, -48)	0.0019	0.0111
Peroxiredoxin-6	P30041	-71 (-85, -44)	0.0016	0.0111
Platelet factor 4	P02776	-71 (-90, -17)	0.0255	0.0465
Histone H1.2	P16403	-70 (-89, -18)	0.0229	0.0431
DNA topoisomerase 1	P11387	-69 (-86, -35)	0.0051	0.0146
Protein S100-A6	P06703	-68 (-82, -41)	0.0016	0.0111
Aflatoxin B1 aldehyde reductase member 2	O43488	-67 (-82, -41)	0.0015	0.0111
Ubiquitin-conjugating enzyme E2 N	P61088	-67 (-82, -39)	0.0021	0.0111
Peroxiredoxin-1	Q06830	-66 (-81, -39)	0.002	0.0111
Ubiquitin+1, truncated mutation for UbB	P62979	-66 (-81, -39)	0.0017	0.0111
Histone acetyltransferase type B catalytic subunit	O14929	-66 (-83, -32)	0.0058	0.0159
Non-histone chromosomal protein HMG-14	P05114	-65 (-84, -27)	0.0093	0.0216
Casein kinase II 2-alpha:2-beta heterotetramer	P68400.P67870	-65 (-80, -37)	0.0021	0.0111

Proteins significantly increased in relative abundance by $\geq 500\%$ (A) or significantly decreased by $\geq 65\%$ (B) following multiple comparisons with adjusted *p*-value of ≤ 0.05 when comparing venous cord blood to arterial cord blood in neonates ≤ 32 weeks of gestation ($N = 12$) using the SomaLogic platform. CI confidence interval.

Here we report that 434 proteins were found in significantly higher relative abundance in the umbilical vein when compared to the artery, consistent with placental release of these proteins into the fetal circulation. Also, 302 proteins were significantly lower in the umbilical vein as compared to the artery suggesting uptake by the placenta. This could represent fetal proteins that are removed by the placenta for metabolism or excretion to the mother or constitute fetal signals to the placenta. Moreover, the abundance of 142 proteins were significantly lower in infant blood collected 48–72 h after delivery as compared to the umbilical vein. On further evaluation of these proteins, 25 proteins had a $\geq 50\%$ lower relative abundance in neonatal blood including beta human chorionic gonadotropin, a hormone which is synthesized by the placenta to stimulate progesterone synthesis by the corpus luteum in order to maintain early pregnancy.⁴¹ Our data

demonstrate that hCG is also secreted into the fetal circulation in the second trimester and decreased by 96% following delivery, representing a good 'internal control' for a protein likely to be synthesized almost exclusively by the placenta.

Critical stages of lung development occur between 20 to 30 weeks of gestation, placing an infant delivered in late second trimester at increased risk of chronic lung disease. Indeed, up to 40% of extremely low birth weight infants develop lung complications.⁴ In our study, 21.7% of patients were affected with moderate to severe chronic lung disease diagnosed at 36 weeks. We speculate that proteins secreted by the placenta may be important for fetal lung development and that the discontinuation of a placental source of these proteins in the infant delivered prematurely may contribute to the development of chronic lung disease. FGF1 may be of importance in this context. First, the

Table 4. Proteins with the largest neonatal-to-umbilical vein blood differences in relative abundance.

Target Full Name	UniProt	% Difference (CI)	P-value	Adjusted P-value
A. Proteins with Lower Relative Abundance in the Neonatal Blood as Compared to the Umbilical Vein				
Human Chorionic Gonadotropin	P01215.P01233	−96 (−97, −92)	<0.0001	<0.0001
Annexin A1	P04083	−82 (−90, −66)	<0.0001	0.0002
Nucleoside diphosphate kinase B	P22392	−81 (−92, −54)	0.0007	0.0071
Thyroid Stimulating Hormone	P01215.P01222	−78 (−90, −53)	0.0004	0.0044
Granulocyte colony-stimulating factor	P09919	−73 (−89, −34)	0.0061	0.0353
Annexin A2	P07355	−71 (−83, −49)	0.0001	0.0017
Chorionic somatomammotropin hormone	P0DML2.P0DML3	−69 (−79, −54)	<0.0001	0.0001
Plasminogen activator inhibitor 1	P05121	−67 (−84, −31)	0.0046	0.0278
Mitogen-activated protein kinase 8	P45983	−63 (−72, −51)	<0.0001	<0.0001
Inosine-5′-monophosphate dehydrogenase 1	P20839	−61 (−72, −47)	<0.0001	0.0001
Activated Protein C	P04070	−59 (−71, −42)	<0.0001	0.0004
Fibroblast growth factor 1	P05230	−56 (−67, −41)	<0.0001	0.0001
Dynein light chain roadblock-type 1	Q9NP97	−55 (−70, −33)	0.0004	0.0046
Fibroblast growth factor 23	Q9GZV9	−55 (−73, −24)	0.0044	0.027
Tyrosine-protein kinase Lyn, isoform B	P07948	−55 (−73, −26)	0.0029	0.0197
Vesicular integral-membrane protein VIP36	Q12907	−55 (−62, −46)	<0.0001	<0.0001
Insulin-like growth factor-binding protein 1	P08833	−55 (−72, −25)	0.0031	0.0207
R-spondin-3	Q9BXY4	−54 (−66, −38)	<0.0001	0.0004
Thrombin	P00734	−54 (−71, −27)	0.002	0.0148
Adenylosuccinate lyase	P30566	−53 (−72, −23)	0.0048	0.0294
Glutathione S-transferase P	P09211	−52 (−70, −23)	0.0041	0.0259
Protein FAM3B	P58499	−52 (−61, −41)	<0.0001	<0.0001
Tyrosine-protein kinase Fer	P16591	−52 (−72, −18)	0.0099	0.0489
Phosphoglycerate kinase 1	P00558	−51 (−70, −18)	0.0082	0.0425
Interleukin-23	P29460.Q9NPF7	−50 (−69, −19)	0.0069	0.038
B. Proteins with Higher Relative Abundance in Neonatal Blood as Compared to the Umbilical Vein				
Trefoil factor 1	P04155	482 (286, 782)	<0.0001	<0.0001
Serum amyloid P-component	P02743	403 (261, 596)	<0.0001	<0.0001
C-reactive protein	P02741	363 (143, 782)	0.0001	0.001
Protein-glutamine gamma-glutamyltransferase E	Q08188	338 (123, 751)	0.0001	0.0018
Complement component C9	P02748	281 (128, 536)	<0.0001	0.0004
Troponin I, fast skeletal muscle	P48788	218 (88, 439)	0.0001	0.0018
Ectonucleoside triphosphate diphosphohydrolase 5	O75356	203 (151, 263)	<0.0001	<0.0001
Midkine	P21741	203 (77, 421)	0.0003	0.0036
Integrin alpha-IIb: beta-3 complex	P08514.P05106	168 (99, 258)	<0.0001	<0.0001
Interleukin-17 receptor B	Q9NRM6	166 (114, 227)	<0.0001	<0.0001
Eotaxin	P51671	160 (92, 253)	<0.0001	<0.0001
C-X-C motif chemokine 13	O43927	153 (91, 236)	<0.0001	<0.0001
Interleukin-1 receptor-like 1	Q01638	148 (60, 281)	0.0002	0.0031
Alpha-1-antichymotrypsin complex	P01011	131 (74, 210)	<0.0001	0.0001
Troponin T, cardiac muscle	P45379	131 (80, 199)	<0.0001	<0.0001
Bone sialoprotein 2	P21815	130 (48, 256)	0.0007	0.0065
Phospholipase A2, membrane associated	P14555	128 (53, 243)	0.0003	0.0039
C-C motif chemokine 28	Q9NRJ3	123 (79, 179)	<0.0001	<0.0001
Peptide YY	P10082	123 (72, 189)	<0.0001	0.0001
Collagenase 3	P45452	122 (44, 239)	0.0008	0.0074
Lymphocyte activation gene 3 protein	P18627	120 (65, 197)	<0.0001	0.0002
Kallikrein-7	P49862	119 (73, 177)	<0.0001	<0.0001
Parathyroid hormone	P01270	116 (61, 189)	<0.0001	0.0003
Alpha-1-antichymotrypsin	P01011	108 (65, 166)	<0.0001	0.0001

Proteins significantly decreased in relative abundance by $\geq 50\%$ (A) or increased by $\geq 100\%$ (B) following multiple comparisons with adjusted p -value of ≤ 0.05 when comparing neonatal blood to venous cord blood in neonates ≤ 32 weeks of gestation ($N = 25$) using the SomaLogic platform. *CI* confidence interval.

Table 5. Proteins significantly decreased in neonatal-to-umbilical venous blood with predicted roles in development.

Protein Name	UNIPRO	Adjusted P Value	% Difference (CI)	Significance
HCG	P01215 P01233	<0.0001	-96 (-97, -92)	<ul style="list-style-type: none"> Synthesized by the placenta to produce progesterone to maintain the corpus luteum⁴¹ Involved in the development of the placenta by stimulating angiogenesis, trophoblast cell differentiation, amongst other functions⁴¹
CD59	P13987	<0.0001	-38 (-44, -30)	<ul style="list-style-type: none"> Knock out mice models suggest this protein protects against ischemic brain damage⁵⁰ Spinal cords of mice deficient in this protein had enhanced inflammation, demyelination, and axonal injury⁵¹ Deficits of this protein are associated with neuritic losses similar to what is seen in Alzheimer's disease⁵²
DCTN2	Q13561	<0.0001	-37 (-43, -29)	<ul style="list-style-type: none"> Present in the nerve growth cone of mice suggesting a role in synapse formation during brain development⁵³
Ephrin-B1	P98172	0.0001	-25 (-32, -17)	<ul style="list-style-type: none"> Knock down of this protein in rats suggest that it functions in the formation of synapses⁵⁴
ERp29	P30040	<0.0001	-49 (-56, -42)	<ul style="list-style-type: none"> In a rat model of spinal cord injury, high expression was associated with an increase in motor neuronal survival and axonal regeneration⁵⁵⁻⁵⁶
FGF1	P05230	0.0001	-56 (-67, -41)	<ul style="list-style-type: none"> Induces early lung development in the embryonic phase of lung development at the stage of budding^{42,43} In an animal model, attenuates TGF-β1-induced pulmonary fibrosis when overexpressed⁴⁴
FGF2	P09038	0.0062	-36 (-50, -19)	<ul style="list-style-type: none"> Knock out models in mice show cerebral cortex defects at birth⁵⁷

HCG human chorionic gonadotropin, DCTN2 Dynactin subunit 2, ERp29 Endoplasmic reticulum resident protein 29, FGF1 Fibroblast growth factor 1, FGF2 Fibroblast growth factor 2, CI Confidence Interval.

relative abundance of this protein was markedly lower in neonatal as compared to umbilical vein blood (56% decrease) and the late second trimester human placenta expressed FGF1 mRNA. These data support the placenta as an important source of FGF1 in the umbilical circulation. Second, FGF1 has previously been shown to induce early lung development at the stage of budding in the embryonic phase of lung development.^{42,43} Moreover, FGF1 has been reported to attenuate TGF-β1-induced pulmonary fibrosis in an animal model,⁴⁴ which is notable given that one of the hallmarks of chronic lung disease in premature infants is increased interstitial fibrosis.⁴⁵ Albeit speculative, we propose that FGF1 secreted from the placenta plays a critical role in fetal lung development and could be developed into a future intervention to attenuate fibrosis in the lungs in premature infants and improve lung related outcomes.

Infants born extremely or very prematurely are at risk for brain damage, including intraventricular hemorrhage (IVH) and periventricular leukomalacia (PVL), which are associated with long-term sequelae including cerebral palsy and learning difficulties.^{6-8,46} Horbar and colleagues found that in infants <1500 g, 6.1% developed IVH grades III and IV and 2.7% were diagnosed with PVL.⁶ In our small cohort, we had a slightly higher percentage (13%) of premature infants with severe IVH. We found several proteins secreted by the placenta into the fetal circulation with known associations to nervous system development, including ERp29, Dynactin subunit 2, FGF2, and CD59 (Table 5). These proteins demonstrated a 36-49% decrease in neonatal blood as compared to umbilical levels, supporting their placental origin. Thus, we speculate that decreased circulating levels of ERp29, Dynactin subunit 2, FGF2, and/or CD59 following premature delivery and loss of the placental circulation, contribute to an increased risk of brain damage in these infants. It is important to note, however, although we have highlighted here proteins that decreased significantly in neonatal blood as compared to the umbilical vein with known roles in brain or lung development in the literature, it is plausible that a smaller but statistically significant decrease in one protein or the combined effect of a decrease in many proteins may be more significant in neonatal development.

Another interesting finding was the 126 proteins that were significantly increased following delivery which may be important in postnatal growth or in dysregulated processes. For example, Midkine (203% increase) is known to be associated with postnatal lung growth⁴⁷ and Eotaxin (160% increase) has been associated with autism in full term infants⁴⁸ as well as is increased in extremely low birth weight infants who die or go on to develop bronchopulmonary disease.⁴⁹

Limitations of this study include that some of the most ill infants were excluded given they required a blood transfusion, which introduced adult proteins before the neonatal blood sample could be taken. Furthermore, given the novelty of our study, the optimal timepoint for the neonatal blood draw is unknown. Although these samples were taken when other samples were needed clinically, it is possible that in order to detect significant decreases in proteins with a longer half-life blood sampling at a later timepoint would be required. Another limitation is that the proteomic platform only evaluates proteins. Many other substances, for example, lipids, carbohydrates, micronutrients, miRNA in exosomes, amongst others may be clinically important substances that are secreted from the placenta and were not analyzed during this study. Lastly for our sub-analysis data, our numbers were small, and data should be considered preliminary.

In conclusion, our data show that a substantial number of placental proteins predicted to be critical for brain and lung development are higher in the umbilical vein compared to the umbilical artery and decrease rapidly in the neonatal circulation following delivery of premature infants. These findings are consistent with the possibility that the human placenta secretes

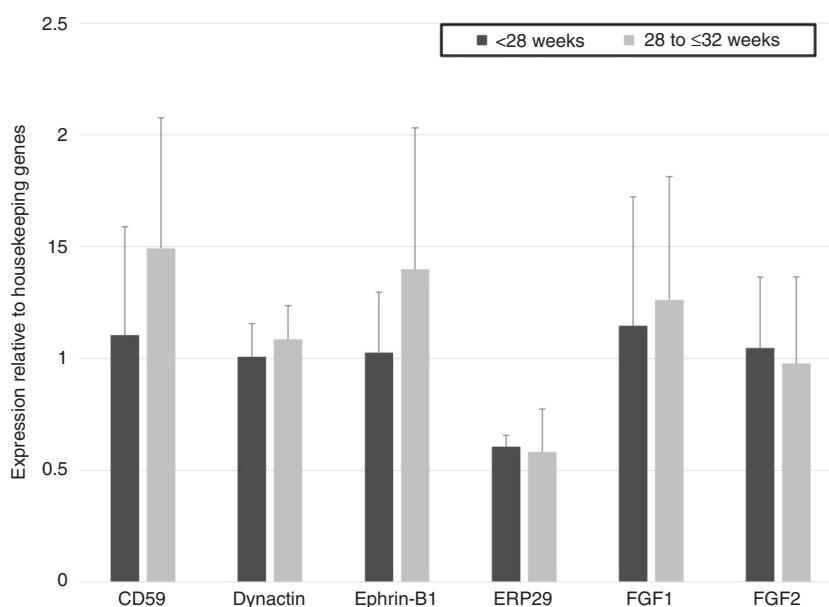


Fig. 2 Placental gene expression. Relative placental expression of genes encoding candidate proteins were expressed at similar levels in extremely and very preterm infants (23 to <28 weeks, $N = 6$; 28 to <32 weeks, $N = 8$) using qPCR. Student t test was used to determine significance between groups. Error bars represents $+1$ SD. *Erp29* endoplasmic reticulum resident protein 29, *Fgf1* Fibroblast growth factor 1, *Fgf2* Fibroblast growth factor 2.

proteins that influence normal development of fetal organs. Importantly, these observations imply that premature infants may be deprived of these critical developmental factors, which likely contribute to poor outcomes. Well-designed animal studies are needed to mechanistically link placental proteins to the development of the fetal brain and lung and to test the hypothesis that administration of selected proteins of placental origin could improve outcomes in prematurity.

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AUTHOR CONTRIBUTIONS

C.S. participated in the design of the experiments, collected the samples, performed mRNA and proteomic analysis, as well as prepared the manuscript. C.P. performed the statistical analysis. T.P. and T.J. designed the experiments, aided in the analysis, and revised the manuscript for critically important intellectual content. All authors read and approved the final manuscript.

CONSENT STATEMENT

IRB #18-0637 approved by the Ethics Committees at Colorado Children's Hospital and University Hospital Colorado. All patients consented to participation with a written informed consent.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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