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Original Research Article

Placental ferroportin protein abundance is associated with neonatal erythropoietic activity and iron status in newborns at high risk for iron deficiency and anemia

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A B S T R A C T

Background: Murine data suggest that the placenta downregulates ferroportin (FPN) when iron is limited to prioritize iron for its own needs. Human data on the impact of maternal and neonatal iron status on placental FPN expression are conflicting.

Objectives: This study aimed to identify determinants of placental FPN protein abundance and to assess the utility of the placental iron deficiency index (PIDI) as a measure of maternal/fetal iron status in newborns at high risk for anemia.

Methods: Placental FPN protein abundance was measured by western blots in placentae collected from 133 neonates born to adolescents (17.4 ± 1.1 y) carrying singletons (delivery gestational age [GA]: 39.9 ± 1.3 wk) and from 130 neonates born to 65 females (30.4 ± 5.2 y) carrying multiples (delivery GA: 35.0 ± 2.8 wk). Placental FPN and the PIDI (FPN:transferrin receptor 1) were evaluated in relation to neonatal and maternal iron-related markers (hemoglobin [Hb], serum ferritin [SF], soluble transferrin receptor [sTfR], total body iron [TBI], hepcidin, erythropoietin [EPO], erythroferrone).

Results: FPN protein was detected in all placentae delivered between 25 and 42 wk GA. Placental FPN protein abundance was associated with neonatal iron and erythropoietic markers (EPO: β: 0.10; 95% confidence interval [CI]: 0.06, 0.35; sTfR: β: 0.20; 95% CI: 0.03, 0.18; hepcidin: β: −0.06; 95% CI: −0.13, −0.0003; all $P < 0.05$). Maternal sTfR was only indirectly associated with placental FPN, with neonatal sTfR as the mediator (β-indirect: 0.06; 95% CI; 0.03, 0.11; $P = 0.003$). The PIDI was associated with neonatal Hb (β: −0.02; 95% CI: −0.03, −0.003), EPO (β: 0.07; 95% CI: 0.01, 0.14), and sTfR (β: 0.13; 95% CI: 0.004, 0.3) and with maternal SF (β: 0.08, 95% CI: 0.02, 0.14), TBI (β: 0.02; 95% CI: 0.009, 0.04), EPO (β: −0.10; 95% CI: −0.19, −0.01), sTfR (β: −0.16: 95% CI: −0.27, −0.06), and hepcidin (β: 0.05; 95% CI: 0.002, 0.11) at delivery (all $P < 0.05$).

Conclusions: Placental FPN abundance was positively associated with neonatal indicators of increased erythropoietic activity and poor iron status. The PIDI was associated with maternal and neonatal iron-related markers but in opposite directions. More data are needed from a lower-risk normative group of females to assess the generalizability of findings.

These trials were registered at clinicaltrials.gov as NCT01019902 and NCT01582802.

Keywords: placenta, iron trafficking proteins, high-risk pregnancy, neonatal anemia, erythropoietic activity

Introduction

The placenta is a fetally-derived organ responsible for transporting all required nutrients, such as iron, to the developing fetus. The fetus accumulates iron over gestation to support its growth and development [1]. Insufficient fetal iron accrual can result in irreversible adverse neurodevelopmental consequences to the newborn [2,3]. In addition to ensuring sufficient iron transport to the fetus, the placenta must also secure sufficient iron to meet its own high metabolic demands [4]. To date, few human studies have investigated placental adaptations to variations in maternal iron availability, and little is known on whose needs predominate when iron supply is limited.

Abbreviations: CI, confidence interval; CRP, C-reactive protein; CV, coefficient of variation; EPO, erythropoietin; ERFE, erythroferrone; FPN, ferroportin; GA, gestational age; Hb, hemoglobin; HIF, hypoxia-inducible factor; ID, iron deficiency; IDA, iron deficiency anemia; IL-6, interlukin-6; p[Fe], placental iron concentration; PIDI, placental iron deficiency index; ppBMI, prepregnancy body mass index; SF, serum ferritin; STB, syncytiotrophoblast; sTfR, soluble transferrin receptor; TBI, total body iron; TFR1, transferrin receptor 1.

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Transport of iron to the fetus occurs through the placental syncytiotrophoblast (STB), and this transfer is thought to be unidirectional [4]. Transferrin-bound iron in maternal circulation is taken up by transferrin receptor 1 (TFR1) expressed on the apical side of the STB. Intracellular nonheme iron can be used or stored by the placenta or can be exported across the fetal side of the STB via the only known nonheme iron export protein, ferroportin (FPN) (Supplemental Figure 1) [1]. Iron must then cross the fetal endothelium to enter fetal circulation, but at present, the process by which this occurs has not been elucidated [5]. Although gaps remain, both TFR1 and FPN are indispensable for fetal iron acquisition as global inactivation of either transporter is embryonically lethal [6–8].

Placental iron uptake and export to the fetus are regulatable processes. Murine [9,10] and human [11–14] data have shown that when maternal iron is limited, the placenta upregulates TFR1 expression, and animal data suggests that the placenta downregulates FPN expression when maternal iron supply is limited [15–18]. At present, human data on the impact of maternal and neonatal iron status on placental FPN expression are limited. The few studies available show contradictory results, with some finding an association of placental FPN protein expression with maternal [12,19,20] or neonatal [20] iron-related markers and others showing no relationship [12,14,15,20]. Variable findings regarding regulation of placental FPN protein expression in humans may be associated differences in the severity of maternal iron deficiency (ID). Additional sources of variability include placental sampling procedures [21–24], variable sample preparation methods [25], and the antibodies utilized to measure FPN [26].

The ratio between FPN and TFR1, referred to as the placental iron deficiency index (PIDI), has been proposed as an index of iron availability in the maternal–fetal environment [15]. The PIDI represents the ratio of iron export to the fetus relative to placental iron uptake from maternal circulation, with a lower PIDI thought to indicate lower placental iron availability. A study in pregnant adults found females with ID ($n = 17$) had a lower PIDI compared to females without ID ($n = 22$) [15]. The PIDI was also lower in females with increased alcohol consumption, which has been associated with a greater risk of iron deficiency anemia (IDA) [27]. The utility of the PIDI as a measure of iron availability during pregnancy has not been tested in relation to neonatal iron status. The aims of this study were to identify determinants of placental FPN protein expression in newborns at higher risk of ID and anemia and to assess the utility of PIDI as a measure of placental and fetal iron availability.

Methods

Study participants

Placental samples were obtained from healthy newborns at greater risk for ID and anemia born to either healthy pregnant adolescents (<19 y) carrying singletons ($n = 133$; adolescent cohort) or to healthy pregnant adults (20–46 y) carrying multiples ($n = 130$ newborns, $n = 65$ mothers; multiples cohort) (Supplemental Figure 2). Participants were recruited from Strong Memorial and Highland Hospitals at the University of Rochester, NY. Eligible newborns in both cohorts had mothers with uncomplicated pregnancies, with no preexisting medical conditions known to impact maternal iron homeostasis (e.g., HIV, diabetes, inflammatory conditions, eating disorders, malabsorption diseases, and hemoglobinopathies) and were not taking any medications known to affect iron status. Studies were approved by the Institutional Review Boards at the University of Rochester and Cornell University. Informed consent was obtained from all participants aged ≥15 y, and both assent and parental consent were obtained from participants aged <15 y in the adolescent cohort.

Detailed methods on demographic data collection have been published [28,29]. Prepregnancy body mass index (ppBMI, in kg/m^2) was calculated based on self-reported weight and height and was categorized as underweight (<18.5), normal weight (18.5–24.9), overweight (25.0–29.9), or obesity (≥30) [30]. All mothers were prescribed prenatal supplements containing 27 mg of iron as part of their prenatal care. Mothers with anemia received additional iron supplements providing 60 to 120 mg/d of iron. Information on self-reported prenatal supplement use has been published [28,29].

Placental collection

In the adolescent cohort, placentae were collected immediately after delivery, and medical staff recorded untrimmed placental weights. In the multiples cohort, placentae were sent to pathology, where trimmed weights and placental dimensions were recorded by placental pathologists. Fused placentae were weighed as a whole, and placental weight per fetus was calculated by dividing total placental mass by the number of newborns. Aliquots of parenchymal tissue were collected from the interior of each placenta across multiple quadrants of the placenta. The aliquots were pooled together, and samples were flash frozen for western blot analysis and quantification of total iron concentration (p[Fe]) or placed in RNA*later* (ThermoFisher Scientific) for mRNA quantification and kept at −80 °C until analysis. Total p[Fe] (reflecting both nonheme and heme iron, reported in μg/g of wet placental weight) was analyzed by inductively coupled plasma-mass spectrometry in a subset of placentae ($n = 95$, multiples cohort; $n = 98$, adolescent cohort) as described [22]. In a subset of placentae from the adolescent cohort only, placental mRNA expression of iron trafficking proteins (TFR1, divalent metal transporter 1, zyklopen, nuclear receptor coactivator 4) and regulatory hormones (hepcidin, erythroferrone [ERFE], and erythropoietin [EPO]) were measured using RT-qPCR as described [19,31].

Western blot analyses

Placental protein lysates were prepared following published methods [32]. Total protein was quantified using a Pierce BCA Assay (ThermoFisher Scientific). For analysis of FPN, samples were prepared in Laemmli sample buffer without reducing agent, and samples were not heated. For analysis of TFR1, samples were prepared in Laemmli sample buffer with dithiothreitol and incubated at 75 °C for 5 min. All samples were resolved in stain-free Bio-Rad 4% to 20% TGX precast gels (Bio-Rad) and exposed to UV light for 1 minute. Gels were then electroblotted onto nitrocellulose membranes (Li-Cor Biosciences, Odyssey). Membranes were imaged for stain-free total protein (loading control) under UV light, blocked with 5% nonfat milk overnight at 4 °C, and probed overnight at 4 °C with primary antibodies specific to each protein of interest. A human anti-human FPN antibody (Amgen) was biotinylated using an EZ-Link Sulfo-NHS-LC-LC Biotin kit (ThermoFisher Scientific) and used to probe for FPN at a dilution of 1:10,000. A mouse anti-human TFR1 antibody (ThermoFisher Scientific) was used to probe for TFR1 at a dilution of 1:2000. Blots were then washed with Tris buffered saline and Tween-20 and incubated with horseradish peroxidase (HRP)-tagged secondary antibodies for 1 h at room temperature (Pierce High Sensitivity NeutrAvidin-HRP [ThermoFisher Scientific] for FPN; HRP-linked anti-mouse IgG [Cell Signaling Technology]

for TFR1). To control for intermembrane variation, a sample of the same control placenta (obtained from an uncomplicated singleton pregnancy) was loaded onto all gels. Membranes were imaged with a FluorChem E system (ProteinSimple), and relative quantification was performed using Alpha View SA, version 3.5.0 (ProteinSimple). Each sample was normalized to stain-free total protein and normalized again to the control placenta. For all participants, placental FPN and TFR1 protein abundance were measured at the same time, utilizing protein lysates from the same placental sample to account for possible variability in these proteins between different sampling sites. The PIDI was calculated for each subject by dividing the normalized expression of FPN by the normalized expression of TFR1. In the adolescent cohort, 132 of the placentas had TFR1 protein abundance previously measured by western blot in the same tissue aliquots [11, 19]. Duplicate measures of TFR1 were significantly correlated ($r = 0.6$, $P < 0.0001$), showing adequate tissue stability over a period of 10 y in tissue kept frozen at −80°C.

Serum collection and biochemical analyses

Nonfasted maternal blood (15 mL) was collected at midgestation (23.8 ± 6.4 wk) and delivery (38.4 ± 2.9 wk), and umbilical cord blood (15 mL) was collected at delivery. Maternal and neonatal hemoglobin (Hb), iron status, erythropoietic indicators (serum ferritin [SF], serum soluble transferrin receptor [sTfR], and serum iron), hormones (hepcidin, EPO, ERFE), and inflammatory markers (C-reactive protein [CRP] and interlukin-6 [IL-6]) were measured in both cohorts. Detailed methods for all biochemical assays have been published [28,29]. Briefly, in both cohorts, Hb was analyzed with a Cell-Dyn 4000 hematology analyzer (Abbott Laboratories). SF and serum sTfR were measured by ELISA (Ramco Laboratories) with an interassay coefficient of variation (CV) of 8% in the multiples cohort and 4% in the adolescent cohort. Serum iron was measured with a graphite furnace atomic absorption spectrophotometer (PerkinElmer AAnalyst 800). Serum EPO was analyzed by immunoassay (Siemens Immulite 2000), and serum ERFE by ELISA (Intrinsic Lifesciences). The interassay CV for ERFE and EPO were 11.4% and 7.5%, respectively. Serum folate and vitamin B12 were analyzed by immunoassay (Siemens Immulite 2000). Hepcidin, CRP, and IL-6 were measured with different assays between the cohorts. In the adolescent cohort, serum hepcidin was measured by ELISA (Intrinsic Life Sciences) with an interassay CV of 12.4%. In the multiples cohort, serum hepcidin was measured by ELISA (Bachem) and had an interassay CV of 12.2%. Standardization of hepcidin across cohorts was performed using the regression slope and intercept values from van der Vroom et al. [33]. Serum CRP was analyzed by immunoassay (Siemens Immulite 2000), and serum IL-6 by Magnetic Multiplex assay (Millipore) in the adolescents. For the multiples cohort, serum CRP and IL-6 were measured by ELISA (R&D Systems). Total body iron (TBI) was calculated as: $\text{TBI (mg/kg)} = -[\log 10(\frac{\text{sTfR}}{\text{SF}}) - 2.8229)/0.1207]$[34]. Data on maternal [28,29,35] and neonatal [36–40] iron status in these cohorts have been published.

Maternal anemia was defined as Hb concentration <11.0 g/dL in the first (<14.0 wk gestation) and third (≥28.0 wk gestation) trimesters, and <10.5 g/dL in the second trimester (14.0–27.9 weeks gestation) [41]. Maternal ID was defined using cutoffs of SF <12.0 μg/L [42], sTfR >8.5 mg/L [43], or TBI <0 mg/kg [34], and IDA was classified as having both ID and anemia. Neonatal anemia was defined as umbilical cord Hb concentration <13.0 g/dL [41].

Statistical analyses

Continuous variables were tested for normality using the Shapiro-Wilk test, and nonnormal variables were logarithmically transformed before analyses. Continuous neonatal, maternal, and placental variables are presented as mean ± SD if normally distributed or as geometric mean and 95% confidence interval (CI) if nonnormally distributed, and categorical variables are presented as percentages. Student's *t* test, Wilcoxon's rank-sum test, and chi-square test were used to test for differences in maternal variables. Linear mixed-effects models were used to test for differences in placental and neonatal variables between cohorts. A maternal identification number was added as a random effect to all models involving the multiples cohort to control for nonindependence of sibling placentas and neonates. Associations of placental FPN or TFR1 protein abundance or the PIDI (dependent variables) with neonatal and maternal descriptive characteristics and iron-related variables (independent variables) were explored using linear mixed-effects in the whole population. A combination of purposive selection and stepwise regression methods was utilized for covariate selection in the whole population analyses. Variables that were of biological relevance and/or significantly different between cohorts were selected; these included study cohort, delivery gestational age (GA), maternal age, maternal self-reported race, maternal self-reported ethnicity, ppBMI, parity, delivery mode, and neonatal birth weight (fully adjusted model). Stepwise regression was then implemented on this subset of variables, and resulting variables from stepwise regression were study cohort and delivery GA (partially adjusted model). Both partially and fully adjusted models are presented. When appropriate, additional covariates were used based on biological relevance. Analyses in the whole population with a statistically significant interaction of study cohort (P-interaction < 0.05) were also evaluated and presented in each cohort separately. A marginal R^2 for mixed-effects models was calculated to estimate the fraction of the variance explained by the fixed effects using published methods [44]. Mediation analyses were conducted using the '*lavaan*' package in R, and the 95% CIs were computed using bootstrap [45]. In the multiples cohort, the percentage of intrauterine variance explained by neonatal and maternal variables was calculated as described [40]. The Benjamini–Hochberg approach was utilized to control for the false discovery rate [46]. *P* values for main analyses are reported with and without adjustment for multiple testing given the exploratory nature of the study. Results of statistical analyses were considered significant if $P < 0.05$. Statistical tests were conducted using R 4.2.2 (The R Foundation for Statistical Computing).

Power calculations.

For the multiple linear regression models, a total sample size of 215 guarantees 80% power to detect associations between relative expression of FPN and TFR1 and the PIDI as dependent variables and maternal or neonatal iron-related markers as independent variables with a small, standardized effect size (Cohen's $f^2 = 0.05$) at a significance level of $P < 0.05$ assuming the partially adjusted model (1 main predictor and 2 covariates). For the correlation analyses, a total sample size of 215 guarantees an 80% power to detect a weak correlation ($r = 0.19$) at an $\alpha = 0.05$. When the correlations are tested separately by cohort, a sample size of 109 ensures an 80% power to detect a small to moderate correlation ($r = 0.3$) at an $\alpha = 0.05$. Based on these calculations, our study sample size is sufficiently powered to detect significant associations for our primary study outcomes. Power calculations were performed using the '*pwr*' package in R [47].

Results

Study population

Data on neonatal, maternal, and placental characteristics are presented in Table 1. Delivery GA of the whole population was 37.5 ± 3.3, with deliveries occurring on average 4.9 wk earlier in the multiples compared to the adolescent cohort. As expected, twin and triplet neonates had a significantly lower birth weight compared with the singletons born to teens, even when adjusting for GA at delivery ($P = 0.004$). Mean ppBMI of the whole population and of each cohort separately fell within the overweight BMI category. Compared with the adolescent cohort, a greater proportion of females in the multiples cohort had prepregnancy obesity (26% compared with 51%, $P < 0.0001$). Most deliveries in the multiples cohort occurred via c-section (72%), whereas most deliveries in the adolescents occurred vaginally (93%). The race and ethnic distribution significantly differed between groups with a greater proportion of neonates whose mother self-reported their race/ethnicity as Black or Hispanic in the adolescent cohort compared with the multiples cohort.

Neonatal and maternal iron status

Data on neonatal and maternal iron-related biomarkers are presented in Supplemental Table 1 and Supplemental Table 2, respectively. The prevalence of neonatal anemia was 23% and did not differ between neonates born to adult females and neonates born to adolescents ($P = 0.3$). The prevalence of maternal anemia in the whole population was 28% at midgestation and 37% at delivery. Given that the populations in this study are higher-risk obstetric populations, the prevalence of maternal anemia was ~ 5 to 8 times higher than the reported US national average (5.4%) in pregnant females [48]. Among anemic pregnant individuals, IDA was present in 50% at midgestation and 45% at delivery. The overall prevalence of maternal ID (SF <12 μg/L) was 34% at midgestation and 24% at delivery. Although these 2 groups were at higher risk of anemia, 55% of females remained nonanemic throughout gestation providing us with opportunities to explore findings in those who did or did not develop anemia across gestation. No differences in cord Hb, representative of the largest iron pool found in newborns [39], or the hepcidin-to-EPO ratio, previously shown to capture the most variance in iron status among neonates [40], were observed between neonates born to pregnant individuals with or

TABLE 1
Neonatal, maternal and placental characteristics in neonates born to females carrying multiple fetuses and to pregnant adolescents carrying singletons

Neonatal characteristics	Whole population	Multiples cohort	Adolescent cohort	*P*
	(*n* = 263)	(*n* = 130)	(*n* = 133)	
GA at delivery, wk	37.5 ± 3.3	35.0 ± 2.8	39.9 ± 1.3	<0.0001
Preterm, % (*n*)[2]	32 (85)	63 (82)	2 (3)	<0.0001
Birth weight, kg	2.8 ± 0.7 (262)	2.2 ± 0.6 (129)	3.3 ± 0.4	<0.0001
LBW, % (*n*)[3]	36 (95/262)	71 (91/129)	3 (4)	<0.0001
Birth length, cm	48.5 ± 4.6 (252)	45.5 ± 4.3 (123)	51.4 ± 2.4 (129)	<0.0001
APGAR (5 min)	8.6 ± 1.3 (255)	8.5 ± 1.6 (128)	8.7 ± 1.0 (127)	0.2
Female, % (*n*)	52 (136)	55 (71)	49 (65)	0.4
Types of multiples				
Twins, % (*n*)	—	68 (88)	—	—
Triplets, % (*n*)	—	32 (42)	—	—
Maternal characteristics	(*n* = 198)	(*n* = 65)	(*n* = 133)	
Age at delivery, y	24.1 ± 7.8	30.4 ± 5.2	17.4 ± 1.1	<0.0001
Black females, % (*n*)[4]	52 (103/198)	26 (17)	65 (86)	<0.0001
Hispanic females, % (*n*)[4]	20 (40/197)	5 (3/64)	28 (37)	0.0002
ppBMI, kg/m^2	27.0 ± 7.6 (197)	29.0 ± 8.6	25.1 ± 5.8 (132)	0.005
Parity ≥ 1, % (*n*)	32 (64/197)	58 (38)	20 (26/132)	<0.0001
C-section, % (*n*)	29 (56/196)	72 (47)	7 (9/131)	<0.0001
Current cigarette use, % (*n*)	8 (15/183)	7 (4/55)	9 (11/128)	0.6
Prenatal supplement use, >2 times/wk % (*n*)[5]	82 (154/188)	92 (55/60)	77 (99/128)	0.08
Placental characteristics	(*n* = 263)	(*n* = 130)	(*n* = 133)	
Fused placentae, % (*n*)	—	75 (93/124)	—	—
Placental wWeight[6]				
Weight/Fetus, g	—	295 ± 92 (124)	605 ± 120 (124)	—
Total Weight/Mom, g	—	671 ± 191 (59)	605 ± 120 (124)	—
PW:BW[6]	—	0.14 ± 0.04 (123)	0.18 ± 0.03 (124)	—
p[Fe], μg/g wet weight	72.6 [57.9 – 91.1] (193)	85.4 [57.8 – 126] (95)	62.1 [48.6 – 79.0] (98)	0.3

Abbreviations: GA, gestational age; LBW, low birth weight; p[Fe], placental iron concentration; ppBMI, prepregnancy body mass index; PW:BW, placental weight to birth weight ratio;

[1]Data presented as mean ± SD, geometric mean [95% CI], or % (*n*). *P* values comparing maternal variables between cohorts are from Student's *t* test, Wilcoxon's rank-sum test, or chi-square test. Linear mixed-effects models were used to test for differences in placental and neonatal variables between cohorts. A maternal identification variable was added as a random effect to all models involving the multiples cohort to control for nonindependence of sibling placentas and neonates.

[2] Preterm defined as birth that occurred prior to 37 wk gestation.

[3] Low birth weight defined as birth weight <2.5 kg.

[4] Self-reported maternal race (Black individual or White individual) and ethnicity (Hispanic individual or non-Hispanic individual).

[5] Self-reported use of prenatal supplements containing on average of 27 mg iron/d or intake of 2 pediatric chewable supplements that provided on average 20 mg iron/d.

[6] Differences between placental weight or placental efficiency (PW:BW) were not compared between cohorts as placental weight measurements in the adolescent cohort were taken before trimming and in the multiples cohort after trimming took place.

without ID, anemia, or IDA at midgestation or delivery in either cohort (all $P > 0.05$).

Placental FPN protein abundance

Placental FPN protein was detected in all placentae delivered between 25 and 42 wk of gestation. Representative blots for FPN protein abundance in both cohorts are shown in Supplemental Figure 3. Mean placental FPN protein abundance in the whole population was 1.37 (95% CI: 1.29, 1.44; range: 0.29–4.04) and was significantly higher in placental samples obtained from the adolescent cohort compared with the multiples cohort (Supplemental Figure 4).

Descriptive predictors of placental FPN protein abundance.

In the whole population, placental FPN protein abundance differed as a function of GA at delivery, with lower FPN protein abundance in placentas delivered later in gestation ($n = 263$, β: −0.04; 95% CI: −0.04, −0.009, $P = 0.01$), and this association remained significant after adjustment for mode of delivery (vaginal or c-section; β: −0.04; 95% CI: −0.07, −0.006; $P = 0.02$). With respect to neonatal variables, placental FPN protein abundance was negatively associated with 5-min APGAR scores ($n = 255$; β: −0.06; 95% CI: −0.10, −0.01; $P = 0.008$), and this association remained significant after adjustment for cord Hb concentrations (β: −0.07; 95% CI; −0.13, −0.02; $P = 0.01$), which is known to be positively associated with APGAR scores [37]. In the whole population, placental FPN protein abundance did not differ as a function of neonatal weight, length, sex, or placental weight (all $P > 0.05$). Placental FPN protein abundance was not associated with p[Fe] ($P = 0.9$). With respect to maternal variables, placental FPN protein abundance was not associated with maternal age ($P = 0.09$), maternal self-reported race ($P = 0.08$) or ethnicity ($P = 0.3$), ppBMI ($P = 0.7$), parity ($P = 0.7$), cigarette smoking ($P = 0.3$), delivery mode ($P = 0.3$), or frequency of prenatal supplement use ($P = 0.1$).

Iron-related predictors of placental FPN protein abundance.

Exploratory analyses were conducted to identify significant neonatal and maternal iron, erythropoietic, and hematological predictors of placental FPN protein abundance. In the pooled neonatal population, indicators of increased erythropoietic activity and poor iron status (higher sTfR and EPO, lower hepcidin), were significantly associated with higher placental FPN protein abundance (Table 2). Ratios between umbilical cord iron-related biomarkers and hormones were explored in relation to placental FPN protein abundance as these may capture coordinated regulation that occurs between erythropoietic drive, hypoxia, and iron availability (Table 2). The neonatal Hb-to-sTfR, Hb-to-EPO, and hepcidin-to-EPO ratios, suggestive of ID in the newborn, had the strongest associations with placental FPN protein abundance, and the Hb-to-sTfR ratio captured the largest proportion of variance in this measure (marginal $R^2 = 0.31$; $P = 0.0002$). Since sTfR is directly related to erythroid precursor mass [49], the associations between placental FPN protein abundance and cord sTfR concentrations and the Hb-to-sTfR and hepcidin-to-sTfR ratios were further adjusted for neonatal birthweight. These associations did not appear to be driven by differences in neonatal birthweight (sTfR: β: 0.21; 95% CI; 0.07, 0.40; P=0.004; Hb-to-sTfR ratio: β: −0.20; 95% CI: −0.44, −0.15; P=0.0001; hepcidin-to-sTfR ratio: β: −0.07; 95% CI: −0.12, −0.02; $P = 0.009$). Associations of placental FPN protein abundance with neonatal iron status markers and ratios remained significant in the fully adjusted models controlling for potential demographic confounders (Table 2). No significant moderating effects of study cohort were observed for any of the neonatal iron-related variables evaluated (all P-interaction > 0.2) (Table 2), and the direction of all regression coefficients was consistent between the 2 cohorts when evaluated separately (Supplemental Table 4).

With respect to maternal variables, placental FPN protein abundance was not associated with any of the maternal hematological, iron status, or erythropoietic variables in the whole population or when evaluated in each cohort separately (Supplemental Table 3). Additionally, placental FPN protein did not significantly differ as a function of maternal ID, anemia, or IDA at midgestation or delivery (Supplemental Figure 5). Significant moderating effects of study cohort were observed for maternal Hb concentrations at midgestation (P-interaction

TABLE 2
Associations between placental FPN protein abundance and neonatal iron-related variables in placentas obtained from neonates born to adult females carrying multiple fetuses and adolescents carrying singletons[1]

Model 1	*n*	β [95% CI]	*P*	FDR *P* value[4]
Hb	196	-0.01 [0.03, 0.02]	0.5	0.6
SF	232	-0.02 [-0.09, 0.06]	0.7	0.8
sTfR	234	0.20 [0.06, 0.35]	0.006	0.02
TBI[2]	232	-0.005 [-0.01, 0.002]	0.2	0.2
Std Hep[3]	231	-0.06 [-0.13, -0.0003]	0.049	0.09
EPO	215	0.10 [0.03, 0.18]	0.004	0.02
ERFE	158	0.03 [-0.02, 0.08]	0.2	0.2
Std Hep:EPO[3]	213	-0.06 [-0.10, -0.02]	0.004	0.02
Std Hep:ERFE[3]	155	-0.04 [-0.08, 0.002]	0.06	0.1
Std Hep:sTfR[3]	231	-0.07 [-0.11, -0.15]	0.01	0.03
Hb:EPO	164	-0.09 [-0.16, -0.02]	0.009	0.02
Hb:sTfR	178	-0.29 [-0.43, -0.14]	0.0002	0.002
Hb:SF	178	-0.0007 [-0.08, 0.06]	0.8	0.9
sTfR:SF	232	0.04 [-0.02, 0.10]	0.2	0.2
Model 2	***n***	**β [95% CI]**	***P***	**FDR *P* value[4]**
Hb	193	-0.008 [-0.03, 0.01]	0.5	0.6
SF	230	-0.02 [-0.10, 0.06]	0.6	0.7
sTfR	232	0.21 [-0.01, 0.002]	0.006	0.03
TBI[2]	230	-0.005 [-0.09, 0.03]	0.1	0.2
Std Hep[3]	229	-0.06 [-0.13, 0.002]	0.06	0.1
EPO	213	0.10 [0.025, 0.18]	0.009	0.03
ERFE	157	0.04 [-0.01, 0.09]	0.2	0.3
Std Hep:EPO[3]	212	-0.06 [-0.10, -0.01]	0.009	0.03
Std Hep:ERFE[3]	154	-0.04 [-0.08, -0.0004]	0.048	0.09
Std Hep:sTfR[3]	229	-0.07 [-0.11, -0.01]	0.01	0.03
Hb:EPO	162	-0.10 [-0.17, -0,02]	0.01	0.03
Hb:sTfR	176	-0.29 [-0.44, -0.13]	0.0003	0.005
Hb:SF	176	-0.0005 [-0.07, 0.07]	0.6	0.7
sTfR:SF	230	0.05 [-0.02, 0.11]	0.1	0.2

Abbreviations: EPO, erythropoietin; ERFE, erythroferrone; FDR, false discovery rate; Hb, hemoglobin; Hep, hepcidin; SF, serum ferritin; Std Hep, standardized hepcidin; sTfR, soluble transferrin receptor; TBI, total body iron.

[1] Regression coefficients and 95% confidence interval (β [95% CI]) are from mixed-effects linear regression models with placental FPN protein abundance as the dependent variable and neonatal iron-related variables as the independent variables. The nonindependence of observations in the multiples cohorts is accounted for by adding a mother identification variable as a random effect. Model 1 is adjusted for study cohort and delivery gestational age (partially adjusted), and model 2 is adjusted for covariates in model 1 + maternal age, maternal self-reported race, maternal self-reported ethnicity, prepregnancy body mass index, parity, delivery mode, neonatal birth weight (fully adjusted).

[2] TBI was calculated with the following equation: $\text{TBI (mg/kg)} = -[\log(\text{sTfR/SF}) - 2.8229)/0.1207]$.

[3] Serum hepcidin was measured with different assays between cohorts and standardized using the regression coefficients from van der Vorm et al. [33].

[4] *P* value corrected for FDR using the Benjamini–Hochberg procedure.

= 0.03) and EPO concentrations at delivery (*P*-interaction = 0.04); however, no significant associations were evident when these analyses were evaluated in each cohort separately (Supplemental Table 3).

Mediating effects of neonatal iron-related predictors on placental FPN protein abundance.

Because maternal and neonatal iron status and erythropoietic indicators are significantly correlated with each other, and based on the strong associations observed between placental FPN protein with neonatal but not maternal iron-related markers, mediation analyses were conducted to evaluate if maternal iron status had indirect effects on placental FPN protein abundance with neonatal iron status as the mediator. Maternal sTfR at midgestation (Figure 1A) and delivery (Figure 1B) had a significant indirect effect on placental FPN expression, and neonatal sTfR had both mediating and independent effects on placental FPN.

The PIDI

Representative blots for TFR1 protein abundance in both cohorts are shown in Supplemental Figure 3. Mean placental TFR1 protein abundance in the whole population was 1.06 (95% CI: 1.02, 1.11; range: 0.31–2.01] and was significantly higher in placental samples obtained from the adolescent compared with the multiples cohort. Mean PIDI did not significantly differ between cohorts (Supplemental Figure 4). Correlations between placental FPN, TFR1, and the PIDI are shown in Figure 2.

Descriptive predictors of the PIDI.

In the whole population, the PIDI was negatively associated with GA at delivery (β: −0.06; 95% CI: −0.08, −0.03; $P < 0.0001$), indicative of lower placental iron availability later in gestation. The PIDI was not associated with neonatal weight, length, sex, APGAR scores, placental weight, or p[Fe] in the cohort as a whole or in each individual cohort (all $P > 0.05$). With respect to maternal variables, a significantly higher PIDI was evident in placentas obtained from mothers with prepregnancy obesity ($n = 69$) compared to mothers without prepregnancy obesity ($n = 177$; β: 0.14; 95%CI: 0.0014, 0.27; $P = 0.04$). The PIDI did not differ as a function of maternal age at delivery, parity, or maternal self-reported race or ethnicity (all $P > 0.05$). Significant moderating effects of study cohort were observed for the associations of the PIDI and delivery mode (c-section compared with vaginal; *P*-interaction = 0.02). In the multiples cohort only, placentas delivered via c-section ($n = 96$) had a significantly higher PIDI compared with placentas delivered vaginally ($n = 34$; β: 0.3; 95% CI: 0.01, 0.35; $P = 0.002$); this difference persisted after adjustment for GA at delivery ($P = 0.04$) and for possible inflammation (CRP: $P = 0.01$; IL-6: $P = 0.08$).

Iron-related predictors of the PIDI.

Figure 3 shows the regression coefficients from the significant associations of neonatal and maternal iron-related and erythropoietic variables with the PIDI (Figure 3A) and with each placental protein evaluated separately (FPN: Figure 3B, TFR1: Figure 3C). In the whole cohort, the PIDI was significantly associated with both neonatal and maternal iron-related indicators, but marked differences in the direction of the relationships were observed. Indicators of **neonatal anemia and increased erythropoietic activity** (low Hb and high EPO and sTfR) were consistently associated with a **higher** PIDI, whereas indicators of **poor maternal iron status or higher erythropoietic activity** (low SF and hepcidin, and high EPO and sTfR) were consistently associated with a **lower** PIDI (Figure 3A). These observations remained constant in the fully adjusted models (Supplemental Figure 6). Similarly, the PIDI was significantly lower in pregnant individuals with ID and IDA compared with those without ID and IDA, respectively (Supplemental Figure 5).

The PIDI and its possible association with neonatal and maternal iron status and regulatory hormone ratios were also explored (Table 3). The PIDI was positively associated with the maternal Hb-to-sTfR and Hb-to-EPO ratios and negatively associated with the neonatal Hb-to-sTfR and Hb-to-EPO ratios. Maternal indicators including TBI, Hb-to-SF, SF-to-sTfR, hepcidin-to-EPO, and hepcidin-to-sTfR ratios were also significant predictors of the PIDI, but these indicators in the neonate were not associated with the PIDI (Table 3).

No differences in the associations of the PIDI with neonatal iron status variables and ratios were observed between neonates in the multiples cohort compared with neonates in the adolescent cohort (all *P*-interaction > 0.1). The relationships between maternal SF and TBI with the PIDI at delivery were only statistically significant in the

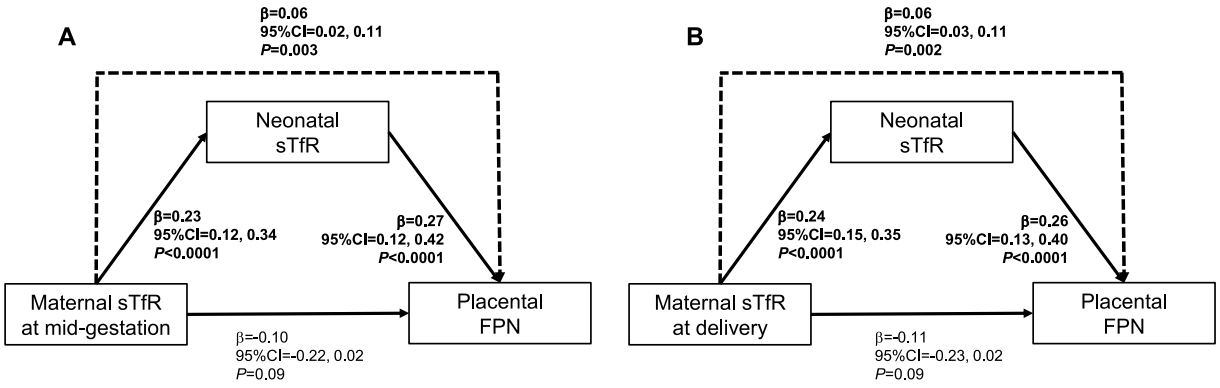

FIGURE 1. Direct and mediating effects of neonatal iron status and erythropoietic activity on placental FPN protein abundance in adult females carrying multiple fetuses and pregnant adolescents carrying a single fetus. Mediation models were utilized to assess direct (solid lines) and indirect (dashed line) effects of neonatal and maternal iron status on placental FPN protein abundance. (A) Maternal serum sTfR at midgestation and (B) maternal serum sTfR at delivery. (A) and (B) show the direct associations (solid line) between maternal sTfR as the independent variable with placental FPN protein abundance and cord sTfR as the dependent variables; the direct associations (solid line) between cord sTfR as the independent variable and placental FPN protein abundance dependent variable; and the indirect association (dashed line) between maternal sTfR at midgestation with placental FPN protein abundance with neonatal sTfR as the mediator. Abbreviations: FPN, ferroportin; sTfR, soluble transferrin receptor.

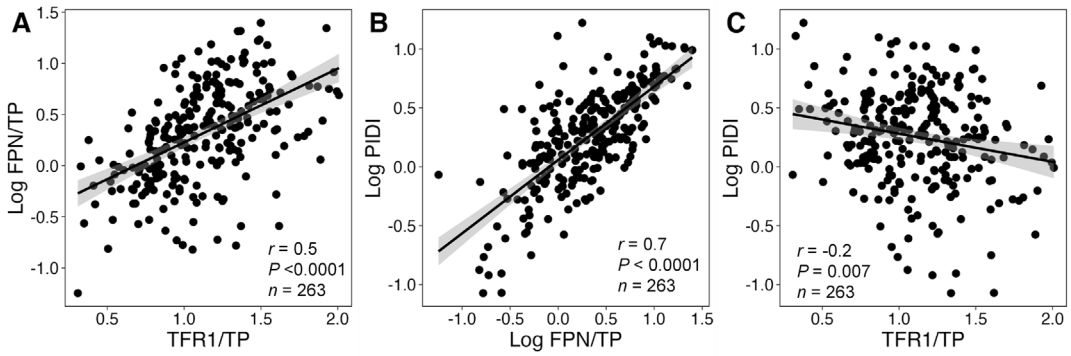

FIGURE 2. Bivariate correlations between the PIDI and placental ferroportin and transferrin receptor 1 protein abundance in placentas obtained from neonates born to adult females carrying multiple fetuses and pregnant adolescents carrying singletons. Linear relationships between placental FPN (relative to the stain-free total protein loading control) and TFR1 and PIDI are shown (A and B), as is the association between TFR1 (relative to the stain-free total protein loading control) and PIDI (C). Variables that were not normally distributed were log transformed prior to analysis. Abbreviations: FPN, ferroportin; PIDI, placental iron deficiency index; TFR1, transferrin receptor 1; TP, stain-free total protein.

multiples cohort, and a weak negative association between the PIDI and maternal Hb at midgestation was observed in the adolescent cohort only (Supplemental Table 5).

Differences in FPN and TFR1 protein abundance and the PIDI between siblings in the multiples cohort

The multiple-birth model was used to explore determinants of placental transporter expression in the context of a fixed maternal environment. Among all maternal and neonatal iron status markers and regulatory hormones evaluated, umbilical cord Hb explained the largest percent of intrauterine variance in the PIDI (10.3%), and cord hepcidin captured the largest percent of intrauterine variance in FPN protein abundance (3.5%). In contrast, maternal SF at midgestation captured the most intrauterine variance in TFR1 protein abundance (4.3%).

Correlations between placental FPN protein abundance and mRNA expression of iron trafficking proteins

Bivariate correlations between FPN protein abundance and mRNA expression of placental iron trafficking proteins and regulatory hormones were explored in a subset of placentae with mRNA data available (n = 109) from the adolescent cohort (Table 4). Placental

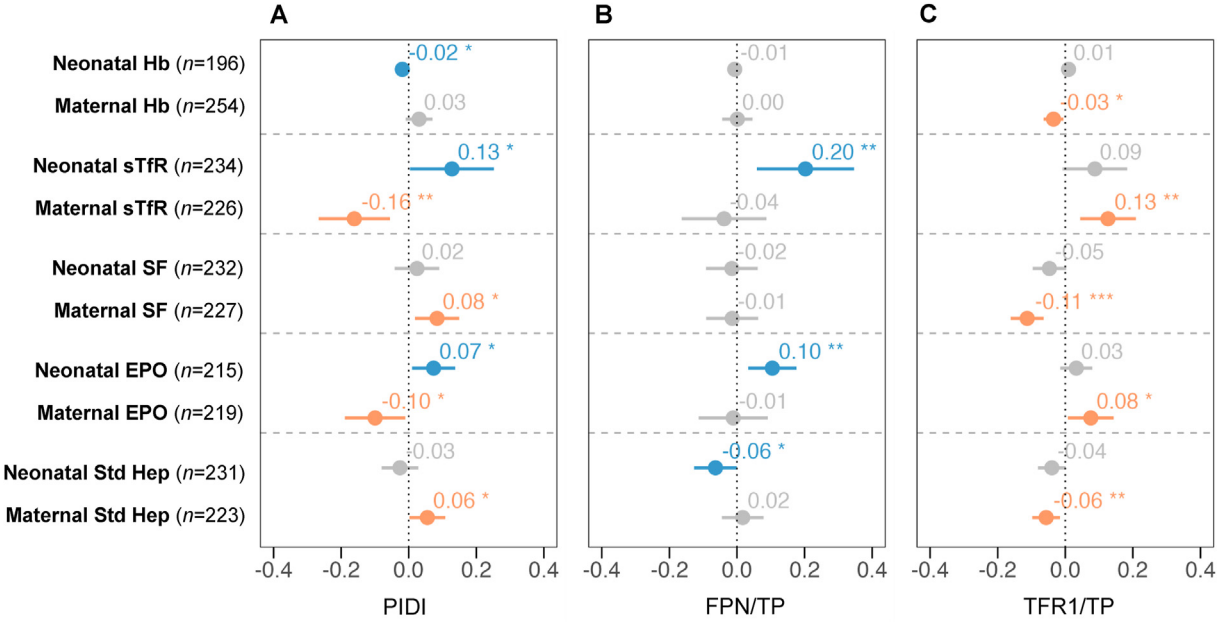

FIGURE 3. Forest plots of regression coefficients from the associations of the PIDI, placental FPN protein and TFR1 protein with maternal and neonatal iron status and erythropoietic markers in adult females carrying multiple fetuses and pregnant adolescents carrying a single fetus. Standardized β coefficients and 95% confidence interval (CIs) from linear mixed-effects regression models of (A) Log PIDI, (B) Log placental FPN/TP, or (C) placental TFR1/TP as the dependent variables and maternal or neonatal iron status markers as the independent variables. Models were adjusted for gestational age at delivery and study cohort. A mother identification variable was included as a random effect to account for the nonindependence of sibling placentas and neonates in the multiples cohort. Hepcidin was measured with different assays between cohorts and standardized using the regression coefficients from van der Vorm et al. [33]. Statistically significant values ($P < 0.05$) are highlighted in blue (neonatal variables) or orange (maternal variables). (*) indicates $P < 0.05$, (**) indicates $P < 0.01$, (***) indicates $P < 0.001$. Abbreviations: EPO, erythropoietin; FPN, ferroportin; Hb, hemoglobin; PIDI, placental iron deficiency index; SF, serum ferritin; Std Hep, standardized hepcidin; sTfR, soluble transferrin receptor; TP, stain-free total protein; TFR1, transferrin receptor 1.

TABLE 3
Associations between the placental iron deficiency index (PIDI) with neonatal and maternal iron status and hormone ratios in adult females carrying multiple fetuses and their neonates and adolescents carrying singletons and their neonates[1]

Model 1	All neonates				All mothers[2]			
	n	β [95% CI]	*P*	FDR *P* value[5]	*n*	β [95% CI]	*P*	FDR *P* value[5]
TBI[3]	232	-0.008 [-0.007, 0.005]	0.8	0.8	226	0.02 [0.009, 0.04]	0.001	0.005
Std Hep:EPO[4]	213	-0.03 [-0.07, 0.004]	0.08	0.2	215	0.04 [0.006, 0.16]	0.03	0.04
Std Hep:ERFE[4]	155	-0.03 [-0.07, 0.007]	0.1	0.2	147	0.01 [-0.02, 0.05]	0.4	0.4
Std Hep:sTfR[4]	231	-0.03 [-0.8, 0.01]	0.2	0.2	222	0.06 [0.2, 0.10]	0.006	0.01
Hb:EPO	164	-0.06 [-0.12, -0.003]	0.04	0.1	213	0.09 [0.006, 0.16]	0.04	0.04
Hb:sTfR	178	-0.15 [-0.3, -0.02]	0.02	0.1	219	0.15 [0.05, 0.25]	0.002	0.006
Hb:SF	178	-0.04 [-0.10, 0.02]	0.2	0.2	220	-0.09 [-0.16, -0.02]	0.01	0.02
sTfR:SF	232	0.007 [-0.05, 0.06]	0.8	0.8	226	-0.08 [-0.13, -0.03]	0.001	0.005
Model 2	*n*	β [95% CI]	*P*	FDR *P* value[5]	*n*	β [95% CI]	*P*	FDR *P* value[5]
TBI[3]	229	-0.001 [-0.01, 0.01]	0.8	0.8	223	0.02 [0.01, 0.04]	0.001	0.004
Std Hep:EPO[4]	211	-0.03 [-0.06, 0.009]	0.1	0.3	212	0.05 [0.005, 0.09]	0.03	0.04
Std Hep:ERFE[4]	153	-0.03 [-0.07, 0.008]	0.1	0.3	145	0.01 [-0.02, 0.05]	0.5	0.5
Std Hep:sTfR[4]	228	-0.02 [-0.07, 0.02]	0.2	0.3	219	0.07 [0.02, 0.11]	0.004	0.007
Hb:EPO	162	-0.06 [-0.10, -0.0004]	0.048	0.2	210	0.10 [0.01, 0.18]	0.02	0.03
Hb:sTfR	229	-0.13 [-0.10, 0.0002]	0.05	0.2	216	0.17 [0.07, 0.3]	0.001	0.004
Hb:SF	176	-0.04 [-0.10, 0.02]	0.2	0.3	217	-0.09 [-0.2, 0.02]	0.02	0.03
sTfR:SF	229	0.006 [-0.05, 0.06]	0.8	0.8	223	-0.09 [-0.1, -0.04]	0.001	0.007

Abbreviations: EPO, erythropoietin; ERFE, erythroferrone; Hb, hemoglobin; FDR, false discovery rate; SF, serum ferritin; sTfR, soluble transferrin receptor; Std Hep, standardized hepcidin; TBI, total body iron.

[1] Regression coefficients 95% confidence interval (β [95% CI]) are from mixed-effects linear regression models with the PIDI as the dependent variable and neonatal or maternal iron-related variables as the independent variables. The nonindependence of observations in the multiples cohorts is accounted for by adding a mother identification variable as a random effect. Model 1 is adjusted for study cohort and delivery gestational age (partially adjusted), and model 2 is adjusted for covariates in model 1 + maternal age, maternal self-reported race, maternal self-reported ethnicity, prepregnancy body mass index, parity, delivery mode, neonatal birth weight (fully adjusted).

[2] Maternal iron status and hormone ratios from samples obtained at delivery.

[3] TBI was calculated with the following equation: $\mathrm{TBI(mg/kg)} = -[\log(\mathrm{sTfR/SF}) - 2.8229)/0.1207]$.

[4] Serum hepcidin was measured with different assays between cohorts and standardized using the regression coefficients from van der Vorm et al. [33].

[5] *P* value corrected for FDR using the Benjamini–Hochberg procedure.

TABLE 4
Correlations between mRNA expression of iron trafficking proteins and ferroportin protein abundance in placentae obtained from neonates born to adolescents carrying singletons[1]

Variable	*n*	*r* [95% CI]	*P*	FDR *P* value[2]
FPN mRNA	109	0.3 [0.2, 0.5]	0.0002	0.002
Hepcidin mRNA	109	-0.3 [-0.4, -0.07]	0.008	0.03
Zyklopen mRNA	109	0.2 [0.05, 0.4]	0.01	0.03
DMT1 mRNA	109	0.2 [0.04, 0.4]	0.02	0.03
TFR1 mRNA	109	0.2 [0.01, 0.4]	0.04	0.06
EPO mRNA	100	0.1 [-0.05, 0.3]	0.1	0.2
ERFE mRNA	100	-0.05 [-0.2, 0.1]	0.6	0.6
NCOA4 mRNA	89	0.05 [-0.2, 0.3]	0.6	0.6

Abbreviations: DMT1, divalent metal transporter, EPO, erythropoietin; ERFE, erythroferrone, FDR, false discovery rate; FPN, ferroportin; NCOA4, nuclear receptor coactivator 4; TFR1, transferrin receptor 1.

[1] Pearson bivariate correlations and 95% confidence interval (*r* [95% CI]). All nonnormally distributed variables were log transformed prior to analyses.

[2] *P* value corrected for false discovery rate using the Benjamini–Hochberg procedure.

FPN protein abundance was positively correlated with placental mRNA expression of FPN, TFR1, DMT1, and zyklopen. Of the 3 regulatory hormones evaluated (EPO, ERFE, and hepcidin), placental FPN protein expression was only significantly correlated with hepcidin mRNA.

Discussion

To our knowledge, this is the largest study to date evaluating both neonatal and maternal determinants of placental FPN protein expression utilizing a validated anti-human FPN antibody [15,26]. In 2 cohorts of neonates at higher risk for ID and anemia, we found higher placental FPN protein abundance to be strongly associated with neonatal erythropoietic activity and iron status but not with maternal iron status and erythropoiesis. In contrast, the PIDI was significantly associated with both neonatal and maternal iron status but in opposite directions. Neonatal anemia/ID status was associated with a higher PIDI, whereas poor maternal iron status was associated with a lower PIDI.

Placental FPN is expressed on the fetal-facing side of the placenta, and therefore, it is directly exposed to fetal regulatory signals [5]. In our study, placental FPN protein was strongly associated with neonatal iron-related markers, with higher FPN protein abundance observed in neonates with higher erythropoietic activity as evidenced by higher umbilical cord EPO and sTfR concentrations and poorer iron status as evidenced by lower cord hepcidin levels. Elevated cord EPO levels have been shown in neonates exposed to hypoxic intrauterine conditions (i.e., intrauterine growth restriction, placental dysfunction, maternal diabetes, maternal smoking) [50–53], and increased fetal sTfR levels result from an increase in overall erythroid activity or under conditions of iron-restricted erythropoiesis [49]. Lower neonatal

Hb-to-sTfR, Hb-to-EPO, hepcidin-to-EPO, and hepcidin-to-sTfR ratios were strongly associated with increased placental FPN protein expression. Lower Hb-to-sTfR and Hb-to-EPO ratios are suggestive of reduced iron availability and ineffective erythropoiesis, and we have previously shown the hepcidin-to-EPO ratio to explain the most variability in newborn iron and hematologic status [40].These data and previous iron status indicator data [38] and stable iron isotope studies [54] suggest that the fetus may have the capacity to regulate iron accretion under conditions of limited iron availability.

FPN is posttranslationally regulated by the main systemic iron regulatory hormone, hepcidin [55]. Animal studies suggest that fetal control of placental FPN expression is limited as knockout of fetal hepcidin did not affect placental FPN protein expression [15]. However, in embryos that overexpress hepcidin [56] or in β-thalassemia models [57], fetal hepcidin appears to have a significant role in modulating placental FPN expression. In our study, we found lower umbilical cord hepcidin to be associated with higher placental FPN protein expression. Additionally, utilizing the multiple-birth model to investigate fetal regulation of placental protein expression, we found that fetal hepcidin explained the largest proportion of variance in placental FPN between neonates sharing a maternal environment. Nonetheless, the proportion of variance in placental FPN abundance explained by fetal hepcidin was relatively small (3.5%). Stronger associations of placental FPN protein with cord EPO and sTfR compared with cord hepcidin were observed, suggesting possible involvement of hepcidin-independent pathways, such as the hypoxia-inducible factor (HIF) system [55], in the regulation of placental FPN protein expression. Under low hepcidin levels, HIF2-α has been shown to stimulate FPN transcript expression in enterocytes [58]. Additional studies are needed to evaluate the contributions of hepcidin-dependent and independent fetal signals in regulating placental FPN expression.

Recent animal studies have shown a marked downregulation of FPN protein expression under conditions of severe ID in pregnant mice [15–18]. We did not see a downregulation of placental FPN protein expression under conditions of maternal ID, anemia, or IDA, and placental FPN protein abundance was not significantly associated with maternal iron regulatory hormones or hematological parameters. In agreement with our findings, other human studies have also not replicated the marked placental FPN decrease seen in mice with severe ID [12,14,15,20]. In one such study, no differences in placental FPN protein abundance were observed between pregnant individuals with (n = 16) and without ID (n = 23) at delivery, even when using a more stringent cutoff to define ID (SF <10 μg/L) [15]. A more recent study found no significant differences in mean placental FPN protein abundance among pregnant individuals with (n = 28) or without (n = 28) IDA (defined as Hb <10.5 g/dL and SF <30 μg/L) [20]. Likewise, another study found no differences in placental FPN protein among pregnant individuals with (n = 18) or without (n = 22) anemia [14]. Differences in placental FPN response in humans compared to animal models may partly be attributed to differences in the degree of severity of ID observed in pregnancy animal models and in pregnant females studied to date. Furthermore, in human pregnancy, females are routinely supplemented with iron, which may alter placental iron acquisition and homeostasis. Finally, in animal studies, placentas are collected before the onset of labor, whereas in human pregnancies, females undergo labor and delivery, which may affect placental protein stability.

Because maternal and umbilical cord iron status biomarkers are correlated, it is important to characterize the direct and indirect effects of maternal and neonatal iron status on placental protein expression. When we evaluated possible indirect effects of maternal iron status and erythropoietic drive on placental FPN protein abundance, we found higher maternal sTfR levels at midgestation and delivery to have an indirect positive effect on placental FPN expression, with neonatal sTfR levels as the mediator. In a study of pregnant individuals with or without prepregnancy obesity carrying a single fetus, placental FPN protein abundance (measured using the same anti-FPN antibody as ours) was found to be positively correlated with maternal sTfR [12], but it is unknown whether these effects are direct or mediated via the fetus. Future studies on iron transport to the fetus should address both maternal and fetal determinants of placental transporters to characterize the regulation of placental iron trafficking more thoroughly.

To date, limited data are available characterizing maternal predictors of the PIDI, and no studies have evaluated the PIDI in relation to newborn iron status. In our high-risk pregnant populations, indicators of poor maternal iron status and increased erythropoietic drive (lower SF, hepcidin, and TBI; higher sTfR and EPO) were strongly associated with a lower PIDI. These findings are consistent with published human and animal data showing a lower PIDI under conditions of maternal ID [15,17] and in pregnancies with alcohol-related increases in maternal hepcidin [27]. Interestingly, the opposite was observed with respect to neonatal iron status variables. We found neonatal anemia and increased erythropoietic activity (as evidenced by lower Hb and higher EPO and sTfR) to be associated with a higher PIDI. Although the PIDI appears to be representative of both maternal and neonatal signals and may serve as a useful research tool, more data are needed to assess how the PIDI as an indicator of iron insufficiency during pregnancy relates to neonatal health outcomes.

In the subset of placentas from the adolescent cohort with placental mRNA data, we found FPN protein to be strongly associated with its upstream FPN mRNA expression, further supporting transcriptional rather than translational regulation of FPN in the placenta. Surprisingly, however, we found a strong negative association between placental FPN protein abundance and placental hepcidin mRNA expression. The direction of the association is consistent with the known inhibiting effects of hepcidin on FPN; however, this result was unexpected based on prior animal [15] and human data that found no significant relationships between placental hepcidin mRNA and other placental iron trafficking proteins [19] or regulatory hormones [31].

Our study has limitations that should be noted. Although our study populations consisted of healthy and uncomplicated pregnancies, newborns and mothers were at increased risk for ID and anemia due to the competing demands that occur when pregnancy coincides with adolescent growth requirements or with multiple fetal-placental units. These unique cohorts provided an opportunity to evaluate placental adaptations that only become evident when maternal iron availability is limited. However, we did not compare our results to a normative group of mothers and neonates. Additionally, maternal iron supplementation (which is a universal recommendation in the United States) and dietary iron intake throughout pregnancy can further alter placental iron homeostasis. In our study, iron supplementation data was self-reported, and we did not account for dietary iron intake. Finally, the mean ppBMI of our population fell within the overweight category, and a large proportion of mothers in our cohort had prepregnancy obesity. ppBMI did not appear to modulate our observations as shown in the models adjusted for ppBMI. Likewise, previous studies have suggested that maternal prepregnancy obesity does not alter placental iron trafficking or protein expression [12,13]. Nonetheless, more data are

needed to understand the impact of maternal obesity and associated complications on placental function because maternal obesity is linked to adverse pregnancy outcomes [59].

A current limitation of this and other studies investigating the placenta is the lack of a standardized protocol for placental tissue collection given the variability in placental morphology and the different cell types present in the placenta. Although it is known that both FPN and TFR1 are produced by syncytiotrophoblasts [5], placental tissue contains several other cell types (such as Hofbauer cells, stromal cells, fibroblasts, among others) [60], and variability in cell type composition between samples is largely unknown. Of note, although placental tissue collection is a concern, it does not appear to have been a significant contributor to observed variability in protein expression as evidenced by the highly consistent and robust results seen for TFR1 in response to maternal ID in humans.

In conclusion, in this large cohort of mothers and newborns at higher risk for ID and anemia, we found newborn iron status and erythropoietic activity to be a strong predictor of placental FPN protein abundance. Our data support available evidence suggesting that the fetus may have the capacity to regulate iron endowment under conditions of limited iron availability, but more data are needed to understand the mechanisms involved in this response. Our findings also highlight the importance of assessing both FPN and TFR1 when investigating placental iron dynamics as these essential iron trafficking proteins are expressed on opposite sides of the placenta and are therefore exposed to different signals. More data are needed to understand how these placental adaptations affect neonatal iron endowment and subsequent development.

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Author contributions

The authors' responsibilities were as follows—AB: designed and performed the experiments, analyzed and interpreted the data, and designed and wrote the manuscript; KOO: designed and conducted the research, analyzed and interpreted data, and wrote the manuscript; RG, EKP, PJK: were responsible for the clinical implementation of the studies and interpretation of the data and with the preparation of the manuscript; EN, TG: assisted with the design of the research and analysis and interpretation of the data and with the preparation of the manuscript; and all authors: read and approved the final manuscript.

Conflict of interest

TG and EN are scientific founders of Intrinsic LifeSciences and Silarus Pharma. All other authors report no conflicts of interest.

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Data availability

Data described in the article, code book, and analytic code will not be made available because of the composition of the patient population and the confidential nature of the data collected.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajcnut.2023.10.022.

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