The Journal of Nutrition
Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

Serum Erythroferrone During Pregnancy Is Related to Erythropoietin but Does Not Predict the Risk of Anemia

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ABSTRACT

Background: Maintaining adequate iron status during pregnancy is important for the mother and her developing fetus. Iron homeostasis is influenced by 3 regulatory hormones: erythropoietin (EPO), hepcidin, and erythroferrone (ERFE). To date, normative data on ERFE across pregnancy and its relations to other hormones and iron status indicators are limited.

Objectives: The objective of this study was to characterize maternal ERFE across pregnancy and at delivery and evaluate the utility of hepcidin, ERFE, and EPO in identifying women with increased iron needs.

Methods: ERFE was measured in extant serum samples collected from 2 longitudinal cohorts composed of women carrying multiple fetuses ($n = 79$) and pregnant adolescents ($n = 218$) at midgestation (~26 wk) and delivery (~39 wk). Receiver operating characteristic curves were generated to characterize the predictive ability of serum ERFE, hepcidin, and EPO and their ratios to identify women at increased risk of iron deficiency and anemia.

Results: In these pregnant women, mean ERFE was 0.48 ng/mL at both ~25 wk of gestation and at delivery. ERFE was positively associated with EPO at midgestation ($\beta = 0.14$, $P = 0.002$, $n = 202$) and delivery ($\beta = 0.12$, $P < 0.001$, $n = 225$) but was not significantly associated with maternal hepcidin at any time point surveyed. Of all hormones measured at midgestation and delivery, EPO was best able to identify women with anemia (AUC: 0.86 and 0.75, respectively) and depleted iron stores (AUC: 0.77 and 0.84), whereas the hepcidin-to-EPO ratio was best able to identify women with iron deficiency anemia (AUC: 0.85 and 0.84).

Conclusions: Maternal ERFE was significantly associated with EPO but was not able to identify women with gestational iron deficiency. At term, the hepcidin-to-EPO ratio, an index that accounts for both iron status and erythropoietic demand, and EPO were the strongest indicators of maternal iron deficiency and anemia. This trial was registered at ClinicalTrials.gov as NCT04517734 (https://clinicaltrials.gov/ct2/show/NCT04517734). *J Nutr* 2021;151:1824–1833.

Keywords: adolescents, hepcidin, iron, multiple births, pregnancy

Introduction

Marked changes in iron physiology occur across pregnancy to help meet the increased maternal, placental, and fetal demand for iron. This increased iron requirement places pregnant women at increased risk of iron deficiency (ID) and iron deficiency anemia (IDA), both of which increase the risk of adverse maternal and neonatal outcomes (1–3). To date, 3 hormones are known to impact iron utilization: erythropoietin (EPO), hepcidin, and erythroferrone (ERFE) (4, 5). These hormones work in concert to regulate iron absorption and erythropoiesis; to date, little is known about the interrelations between these hormones in human pregnancies.

In the United States, 16% of pregnant women are estimated to develop ID (6). The prevalence of ID increases ~4-fold from early to late gestation (7). ID is typically identified based on measures of depleted serum ferritin (SF) or increased soluble transferrin receptor (sTfR) concentrations. SF has been found to correlate with both tissue iron reserves and bone marrow iron in pregnant women (8), but SF is also an acute-phase protein, limiting utility of this indicator when infection or inflammation is present (9–12). Numerous studies have attempted to adjust SF concentrations for concurrent inflammation (11–15), but these corrections are not well established in pregnant women. sTfR has also been used to classify ID in pregnant women (16, 17),

Manuscript received November 19, 2020. Initial review completed January 12, 2021. Revision accepted March 15, 2021.
First published online May 19, 2021; doi: https://doi.org/10.1093/jn/nxab093.

but this indicator may also be impacted by inflammation (18, 19).

Given the limitations associated with the interpretation of current iron status biomarkers during pregnancy, additional insight into maternal iron demand during this key life stage may be obtained by measuring hormones that respond to hypoxia, iron status, and erythropoietic demand. EPO is produced by the kidneys in response to hypoxia and stimulates the mobilization of iron in support of erythropoiesis (20). During pregnancy, EPO concentrations increase compared with values observed in nonpregnant women (21–23), but less is known regarding its utility as an indirect index of iron demand. Hepcidin is a hepatic hormone that functions to reduce cellular nonheme iron export (24). During pregnancy, hepcidin concentrations decrease (25) and animal data suggest this hormone is downregulated early in gestation (26, 27). To date, the ability of hepcidin to identify pregnant women with ID has been assessed (28–32); however, this hormone also functions as an acute-phase protein, limiting its utility for this purpose when inflammation is present. Erythroferrone was recently discovered and found to be produced by erythroblasts in response to EPO. ERFE increases circulating iron concentrations by decreasing hepatic expression of hepcidin (4, 33). Unlike hepcidin, ERFE concentrations are not thought to be directly affected by inflammation (34). A validated ERFE assay was developed in 2017 (35), but ERFE concentrations have yet to be characterized in healthy pregnant women or in animal models of pregnancy, as well as its utility in identifying women with increased iron needs.

To address these gaps, the primary objective of this article was to characterize ERFE concentrations across gestation in women at increased risk of ID and IDA, and to evaluate the relation between ERFE and 2 other iron regulatory hormones (EPO and hepcidin). All 3 regulatory hormones are needed to maintain iron homeostasis in response to iron demands, hypoxia, and erythropoietic activity. Ratios between these hormones may provide additional benefit as they capture hormonal responses that are driven by >1 regulatory pathway. Therefore, the secondary objective was to evaluate ratios between these hormones and their predictive ability to identify women at increased risk of developing anemia, ID, or IDA during pregnancy.

Methods

Participants

ERFE was measured in extant serum collected from 2 pregnancy cohorts. The first cohort consisted of pregnant adolescents recruited from the Rochester Adolescent Maternity Program (RAMP) in Rochester, New York, between 2006 and 2012. The second cohort included women carrying multiple fetuses who were recruited from Strong Memorial Hospital and Highland Hospital in Rochester, New York, from 2011 to 2014 (**Supplemental Figure 1**). Pregnant women and adolescents were excluded if they had HIV, eating disorders, pre-existing diabetes, malabsorption disease, or other medical conditions known to potentially impact iron homeostasis. Informed written consent was obtained at baseline from all participants >14 y of age, and parental consent and adolescent assent were obtained from adolescents ≤14 y of age. Both studies were approved by the institutional review boards of the University of Rochester and Cornell University. Descriptive data and data on iron status from the pregnant adolescents (36–40) and women carrying multiple fetuses (40–43) have previously been published. The study was registered at clinicaltrials.gov as NCT04517734 (https://clinicaltrials.gov/ct2/show/NCT04517734).

Demographic information was self-reported upon entry to the study, and a prenatal supplement questionnaire was completed to assess type and frequency of prenatal supplement use during pregnancy. For both cohorts, maternal anthropometric information was recorded at each study visit. Prepregnancy BMI (ppBMI) was classified using CDC guidelines as either underweight (in kg/m^2; ppBMI <18.5), normal weight (ppBMI ≥18.5 and <25), overweight (ppBMI ≥25 and <30), or obese (ppBMI ≥30) (44). Additionally, gestational weight gain (GWG) categories were classified using Institute of Medicine categories with adjustment for gestational age at delivery. In the adolescent cohort, recommended weight gain was 12.7–18.1 kg for underweight women, 11.3–15.9 kg for normal-weight women, 6.8–11.3 kg for overweight women, and 5–9.1 kg for obese women. In the multiples cohort, recommended GWG was 22.7–28.1 kg for underweight women, 16.8–24.5 kg for women of normal weight, 14.1–22.7 kg for overweight women, and 11.3–19.1 kg for obese women (44). Gestational age was determined based on self-reported menstrual history and sonogram data or by date of in vitro fertilization, when applicable, in the multiples cohort. If self-reported menstrual history and sonogram data differed by >10 d, ultrasound estimates were used to determine gestational age. All participants were prescribed a standard iron-containing prenatal supplement as part of routine prenatal care, and compliance has been previously reported (38, 43). Preeclampsia was diagnosed in 18 women (22%) of the multiples cohort and in 14 women (6%) of the pregnant adolescent cohort. Gestational diabetes developed in 4 women (5%) from the multiples cohort and 2 teen-aged females (1%) in the pregnant adolescent cohort.

Serum collection and biochemical analysis

Nonfasted maternal blood (15 mL) was collected from women at midgestation (~26 wk, n = 226) and when admitted to the hospital for delivery (~39 wk, n = 333). As this was a convenience sample, the number of blood samples collected and the timing of collection across gestation varied (**Supplemental Figure 2**). In both cohorts, whole blood was sent to the University of Rochester core laboratory for assessment of hemoglobin (Hb) concentration using a Cell-Dyn 4000 hematology analyzer (Abbott Diagnostics). Anemia across pregnancy was defined for Caucasians as Hb concentration <11.0 g/dL in the first and third trimesters and <10.5 g/dL in the second trimester and for African Americans as <10.2 g/dL in the first and third trimesters and <9.7 g/dL in the second trimester (45). Remaining blood samples were centrifuged for 10 minutes at 1100 - 1300 g in 4°C to seperate serum, and the serum was stored at −80°C until analysis.

Serum ERFE was measured using a validated ELISA (Intrinsic Lifesciences). Although the assay states the kit has a lower limit of detection (LOD) of 1.5 ng/mL, it provides quantitative measures of ERFE down to 0.001 ng/mL, and the CV for the values <1.5 ng/mL was <10%. As such, absolute values of this hormone were utilized for statistical analyses. Longitudinal samples from individuals were run on the same plate, and a pooled control serum was included on every plate to monitor the interassay CV, which was 11.4%. Methods used to analyze the other iron status biomarkers have been published in detail (36, 38, 43). In brief, iron status biomarkers and regulatory

Supported by the US National Institutes of Health (NIH) National Institute of Child Health and Development (NICHD) grant 1R21HD098864, the NIH National Institute of Digestive and Kidney Diseases (NIDDK) grant T32-DK007158, the Gerber Foundation, and USDA grants 2005-35200-15218 and 2009-35200-05171.

Author disclosures: EN and TG are stockholders and consultants for Intrinsic LifeSciences, LLC. All other authors report no conflicts of interest. The Gerber Foundation had no conflict of interest in the study and was not involved in the study design or interpretation of the data.

Supplemental Figures 1–3 and Supplemental Tables 1–3 are available from the "Supplementary data" link in the online posting of the article from the same link in the online table of contents at https://academic.oup.com/jn/.

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Abbreviations used: BMP, bone morphogenic protein; CRP, C-reactive protein; EPO, erythropoietin; ERFE, erythroferrone; GWG, gestational weight gain; Hb, hemoglobin; ID, iron deficiency; IDA, iron deficiency anemia; LOD, limit of detection; ppBMI, prepregnancy BMI; ROC, receiver operating characteristic curve; SF, serum ferritin; sTfR, soluble transferrin receptor; TBI, total body iron.

hormones were measured within each cohort at the time of sample collection, at times samples from both cohorts were run together. Serum EPO was measured by immunoassay (Siemens Immulite 2000) with an interassay CV of 7.5%. SF and serum sTfR were measured by ELISA as previously described (36, 43). ID was defined using the cutoffs of either SF <12 μg/L or sTfR >8.5 mg/L, and IDA was classified in those with anemia and ID. Total body iron (TBI) was calculated using SF and sTfR as previously described (46). Depleted iron stores were defined as TBI ≤0 mg/kg. Serum iron was measured by atomic absorption spectrophotometry (Perkin Elmer AAnalyst 800). Serum folate and serum vitamin B-12 were measured by an Immulite 2000 immunoassay system (Siemens Healthcare). Folate insufficiency was defined as folate concentrations <6.8 nmol/L and vitamin B-12 insufficiency was defined as vitamin B-12 concentration <148 pmol/L (47, 48). Serum hepcidin, C-reactive protein (CRP), and IL-6 were measured using different assays in each cohort, so quantitative comparisons between cohorts cannot be made. For the adolescent cohort, hepcidin was measured with an ELISA from Intrinsic Lifesciences; the LOD was 5 ng/mL and the interassay CV for this assay was 12.4%. Hepcidin values below the LOD were assigned a value of 2.5 ng/mL for analysis purposes, and 10% of women studied had hepcidin values that fell below the assay LOD. In the multiples cohort, hepcidin was measured with an ELISA from Bachem that had an LOD of 0.39 ng/mL. Women with values below the LOD had a hepcidin value of 0.195 ng/mL assigned for analysis purposes. In the multiples cohort, 23% of women had values that fell below the LOD. The interassay CV for the Bachem hepcidin assay was 12.2% (43). In the adolescents cohort, IL-6 was measured by a multiplex assay (Millipore Magnetic Multiplex) and CRP was measured by immunoassay (Siemens Immulite 2000) (36). For the multiples cohort, IL-6 and CRP were measured by ELISA from R&D Systems (43).

Statistical analysis

Maternal characteristics are presented as means ± SDs or percentages for continuous and categorical outcomes, respectively. The Shapiro-Wilk test was used to assess normality of data, and nonnormal variables were log transformed to achieve normality. Student's *t* test and ANOVA were conducted to test whether normally distributed variables differed by maternal cohorts, and Wilcoxon's rank-sum test was used to test statistical differences between nonparametric variables. Chi-square test of independence was used for analyses of differences between categorical variables between cohorts. Indicators measured with different assay techniques between cohorts (IL-6, hepcidin, and CRP) were converted into *z* scores within each cohort and the *z* scores were used to assess associations between these indicators in the group as a whole. Pearson correlation coefficients were calculated for bivariate relations between iron status indicators. The significance of the relation remained the same after controlling for gestational age at each time point and study population, so only unadjusted data are presented. Bartlett's test was used to evaluate potential differences in the ERFE variance between cohorts. Random slope and intercept models were used to assess longitudinal changes in ERFE while controlling for study population. Multiple regression was used to determine predictors of maternal ERFE concentration at midgestation and delivery. In multiple regression models, variables with bivariate correlation *P* values <0.2 were tested simultaneously and eliminated by backward selection until only statistically significant predictors remained, all while controlling for study population. Statistical analyses were performed using JMP 14.0 (SAS Institute, Inc,). STATA 16 (StataCorp LLC) was used to create mediation models to assess interrelations between iron regulatory hormones. Receiver operating characteristic curves (ROCs) were generated as sensitivity (%) versus 100% − specificity (%) for varying indicator concentrations. The AUC for each indicator was calculated as a test for identifying anemia, IDA, and depleted iron stores. An AUC of 1 indicates perfect accuracy, an AUC of 0.5 indicates that the indicator has similar accuracy to random chance, and an AUC <0.5 indicates the indicator performs worse than random chance (49). The Youden J statistic (sensitivity + specificity − 1) was used to determine the concentration at which the hormones' differentiating ability was optimized.

Results

Maternal characteristics

Characteristics of the 2 cohorts are presented in **Table 1.** Women in the multiples cohort were significantly older, entered pregnancy with a higher parity, had a higher ppBMI, higher GWG, and a significantly shorter gestational period compared with the adolescent cohort. Overall, 15% of women were anemic at midgestation and 24% were anemic at delivery. Of women who were anemic, 74% had IDA at midgestation and 53% had IDA at delivery. At midgestation and delivery, the prevalence of anemia was significantly higher in the multiples cohort (both $P < 0.01$). Additionally, 15% of women had low body iron (TBI <0 mg/kg) at both midgestation and delivery. None of the participants studied were vitamin B-12 insufficient at midgestation, but at delivery, 11% of those screened were found to be vitamin B-12 insufficient. Iron status of the women at midgestation and delivery is presented in **Supplemental Table 1.**

Maternal ERFE and hormone concentrations during pregnancy

Maternal ERFE, EPO, and hepcidin values and the mean concentration ratios between EPO, hepcidin, and ERFE are presented in **Table 2.** Ratios between these regulatory hormones were explored to capture adaptive responses to iron status (hepcidin) and erythropoietic drive and hypoxia (EPO and ERFE). Serum for ERFE analysis was available from 88% of the original cohort. There were no significant differences in subject characteristics or iron status indicators between those with samples available for ERFE measures and those without ERFE measured at either midgestation or at delivery. In the entire cohort, the geometric mean ERFE concentration did not significantly differ between the midgestation or delivery time points, and ERFE averaged 0.48 ng/mL (95% CI: 0.37, 0.61 ng/mL) at midgestation (26 ± 4 wk) and 0.48 ng/mL (95% CI: 0.39, 0.60 ng/mL) at delivery (39 ± 3 wk). At midgestation, there were no significant differences in mean ERFE concentrations between the adolescent and multiples cohorts ($P = 0.44$). At delivery, there were significant differences in mean ERFE concentrations between the adolescent and multiples cohorts ($P = 0.04$). Maternal ERFE did not differ as a function of race ($P > 0.5$) or mode of delivery ($P = 0.3$).

ERFE's association with hormones and iron status biomarkers

In the combined cohort, ERFE concentrations at midgestation were significantly elevated in women with depleted iron stores ($P < 0.01$). A similar finding was evident at delivery (TBI <0 mg/kg, $P = 0.01$; SF <12 μg/L, $P = 0.2$). However, after adjusting for IL-6, there was a higher concentration of ERFE in women with ID (SF <12 μg/L) at delivery ($n = 54$; $P = 0.05$).

In the combined study population at midgestation, ERFE concentrations were significantly higher in anemic women ($n = 27$) compared with nonanemic women ($n = 155$) ($P < 0.01$). However, at delivery, differences in ERFE between anemic ($n = 52$) and nonanemic women ($n = 169$) were not significant ($P = 0.09$).

Bivariate correlations between ERFE and iron status indicators as well as regulatory hormones are presented in **Supplemental Table 2.** In the multiples cohort, ERFE was significantly inversely associated with Hb (~25 wk, $P = 0.03$; ~35 wk, $P = 0.002$) and hematocrit (~25 wk, $P = 0.05$;

TABLE 1 Characteristics of women carrying multiples and pregnant adolescents[1]

Variable	Whole population ($n = 338$)	Multiples cohort ($n = 83$)	Adolescent cohort ($n = 255$)
Maternal age, y	20.6 ± 6.2	30.3 ± 5.1*	17.4 ± 1.1
Race			
African-American, %	60	25*	71
Ethnicity			
Hispanic, %	20	6*	24
Maternal ppBMI,[2] kg/m^2	25.7 ± 6.5	28.2 ± 8.1*	24.9 ± 5.6
Underweight, %	6	5	7
Normal, %	51.5	42	54
Overweight, %	20.5	20	21
Obese, %	22	33	18
Gestational weight gain,[3] kg	17.5 ± 8.0	19.6 ± 9.2*	16.9 ± 7.5
Less than recommended, %	19	33*	15
Recommended, %	28	43*	22
More than recommended, %	53	24*	63
Parity ≥1, %	28	59*	17
Mode of delivery			
Cesarean, %	30	72*	16
Use of cigarettes			
Currently, %	8	8	7
Prenatal supplement use, %			
Everyday	54	82*	46
2–5 times/wk	21	11*	24
< 2 times/wk	25	7*	30
Types of multiples, %			
Twins	77.1	77.1	—
Triplets	21.7	21.7	—
Quadruplets	1.2	1.2	—

[1] Values are means ± SDs or percentages. *Denotes a significant difference between cohorts at $P < 0.05$. ppBMI, prepregnancy BMI.
[2] ppBMI was classified using CDC guidelines (44): underweight classified as BMI (in kg/m^2) <18.5; normal weight was classified as BMI ≥18.5 and <25; overweight was classified as BMI ≥25 and <3; obese was classified as BMI ≥30.
[3] Gestational weight gain categories were classified using Institute of Medicine categories with adjustment for gestational age at delivery (44). For the adolescent cohort, recommended weight gain was 12.7–18.1 kg for underweight women, 11.3–15.9 kg for normal-weight women, 6.8–11.3 kg for overweight women, and 5–9.1 kg for obese women. For the multiples cohort, recommended gestational weight gain was 22.7–28.1 kg for underweight women, 16.8–24.5 kg for women of normal weight, 14.1–22.7 kg for overweight women, and 11.3–19.1 kg for obese women.

~35 wk, $P = 0.01$) and thus, in this cohort, ERFE was significantly higher in anemic women at both midgestation ($n = 71$; $P = 0.001$) and at delivery ($n = 49$; $P < 0.01$). In contrast, in the adolescent cohort, there were no significant relations between ERFE and Hb (~26 wk, $P = 0.8$; ~39 wk, $P = 0.3$) or hematocrit (~26 wk, $P = 0.6$; ~39 wk, $P = 0.26$) and ERFE concentrations did not significantly differ between anemic and nonanemic adolescents at midgestation ($n = 111$; $P = 0.2$) or delivery ($n = 174$; $P = 0.7$). Similar to the relation between ERFE and Hb, the relation between EPO and Hb was stronger in the multiples cohort (midgestation: multiples $\beta = -0.25$, $P < 0.001$; adolescent $\beta = -0.21$, $P < 0.001$; delivery: multiples $\beta = -0.31$, $P < 0.001$; adolescents $\beta = -0.21$, $P < 0.001$). Additionally, in the adolescent cohort, ERFE was positively associated with IL-6 concentrations ($P < 0.01$ midgestation; $P = 0.05$ delivery),

TABLE 2 Serum iron regulatory hormone concentrations and their ratios in adolescents and women carrying multiples during pregnancy[1]

	Multiples cohort		Adolescent cohort	
Variable	Midgestation	Delivery	Midgestation	Delivery
ERFE, ng/mL	0.6 [0.4–0.8]$^{\#}$ (72)	0.9 [0.6–1.3]* (49)	0.5 [0.3–0.6] (139)	0.4 [0.3–0.5] (185)
EPO, mIU/mL	29.8 [25.6–34.7]$^{\#}$ (72)	34.7 [27.5–43.9]* (59)	28.3 [26.1–30.8] (143)	26.5 [24.2–28.9] (202)
Hepcidin,[2] ng/mL	1.3 [0.8–1.9]$^{\#}$ (72)	2.3 [1.6–3.2] (60)	20.7 [18.0–23.8] (144)	24.2 [20.9–28.2] (205)
Percentage undetectable[3]	23$^{\#*}$ (17)	3 (2)	5 (7)	10 (20)
H:EPO[2]	0.04 [0.03–0.07] (72)	0.06 [0.04–0.10] (58)	0.7 [0.6–0.9] (141)	0.9 [0.8–1.1] (199)
H:ERFE[2]	2.3 [1.3–4.0] (71)	2.5 [1.4–4.5] (48)	47.0 [32.4–68.0] (134)	57.70 [42.1–79.1] (180)
ERFE:EPO	0.02 [0.01–0.02] (70)	0.03 [0.02–0.03]* (48)	0.02 [0.01–0.02] (132)	0.01 [0.01–0.02] (177)

[1] Values are geometric means [95% CIs] (n) for transformed variables or percentages (n). $^{\#}$Significant difference between midgestation and delivery within a cohort; *Significant difference compared with the adolescent cohort ($P < 0.05$). EPO, erythropoietin; ERFE, erythroferrone; H, hepcidin.
[2] Differences between cohorts in hepcidin, H:EPO, H:ERFE concentrations were not assessed as hepcidin was measured with different assays between cohorts.
[3] Hepcidin limit of detection for the adolescent cohort was 5 ng/mL and for the multiples cohort was 0.39 ng/mL.

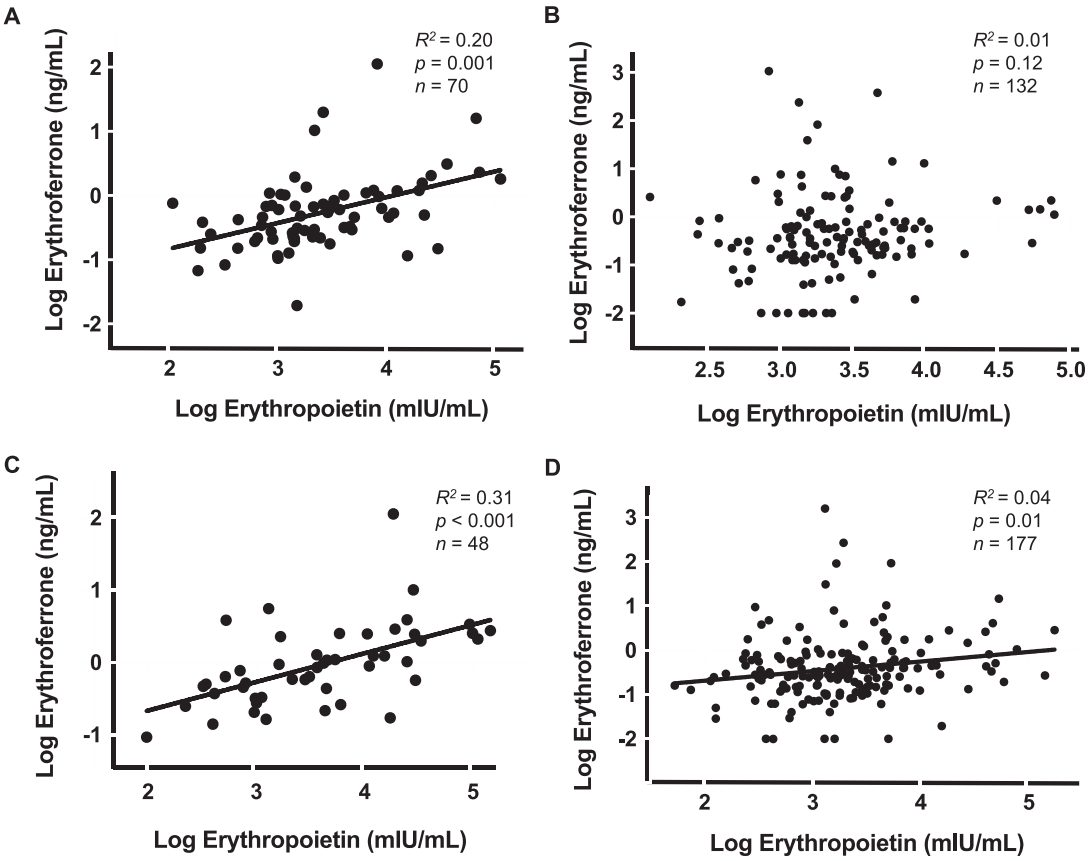

FIGURE 1 Correlations between ERFE and EPO in women carrying multiples and pregnant adolescents. Bivariate correlations between EPO and ERFE in the multiples cohort at midgestation (A) and delivery (C). Bivariate correlations between EPO and ERFE in the adolescent cohort at midgestation (B) and delivery (D). EPO, erythropoietin; ERFE, erythroferrone.

whereas no significant relation between IL-6 and ERFE was observed in the multiples cohort at either midgestation ($P = 0.8$) or delivery ($P = 0.9$).

ERFE was significantly positively associated with EPO at midgestation and delivery in the group as a whole (Supplemental Table 2). When evaluated within each cohort, the relation between EPO and ERFE was only significant in the multiples cohort (Supplemental Table 2, **Figure 1**). Although ERFE is known to decrease hepatic hepcidin expression, there were no significant associations between ERFE and hepcidin at either midgestation ($P = 0.30$) or at delivery ($P = 0.93$) in the group as a whole or within each individual cohort. We did, however, observe an inverse association between EPO and hepcidin (38, 43), and since animal studies have found that ERFE plays a mediating role between EPO and hepcidin (4), potential relations between EPO and hepcidin at midgestation and delivery were explored using mediation analysis. In the combined and individual cohorts, ERFE did not mediate the relation observed between EPO and hepcidin at midgestation or delivery (**Figure 2**). Adjusting for inflammation (CRP or IL-6), ppBMI, or Hb and SF did not alter the significance of any of the relations evaluated.

Determinants of ERFE

Using all available biomarker data, an integrative model was developed to capture determinants of the variance in ERFE at both midgestation and delivery (**Supplemental Table 3**). In the combined cohort, maternal sTfR was the strongest determinant of ERFE and explained 15% of variance. Only minimal improvements (3%) in the model fit were obtained after including EPO and hepcidin. At delivery, sTfR alone explained 13% of variance seen in maternal ERFE ($n = 231$; $\beta = 0.31$, $P < 0.001$), and the addition of EPO and hepcidin to the model only increased this value to 17%. Because of the previous differences in associations noted between cohorts, we explored determinants of the variance in ERFE by cohort (Supplemental Table 3). Consistent with the greater ERFE variability evident in the adolescent cohort, <30% of variance in ERFE was captured by measured variables compared with 40–50% in the multiples cohort.

Longitudinal changes in ERFE

Possible differences in the pattern of change in ERFE across gestation in the cohort as a whole were explored with longitudinal analysis using a random intercept and slope model. In the combined cohort, ERFE increased between 8 and 42 wk of gestation by 0.019 ng/mL per week (β: 0.019; 95% CI: 0.004, 0.035). In the multiples cohort, a greater increase in ERFE across gestation was observed in women carrying triplets/quadruplets ($n = 19$; $\beta = 0.056$, $P = 0.06$) compared with that observed in the women carrying twins ($n = 19$; $\beta = 0.012$, $P = 0.06$).

The significant increase in ERFE across gestation was driven by the multiples cohort as ERFE concentrations did not significantly change across pregnancy in the adolescent cohort (**Supplemental Figure 3**). This may have been impacted by significantly greater variance in ERFE found in the adolescent cohort at both midgestation (Bartlett's $P < 0.001$)

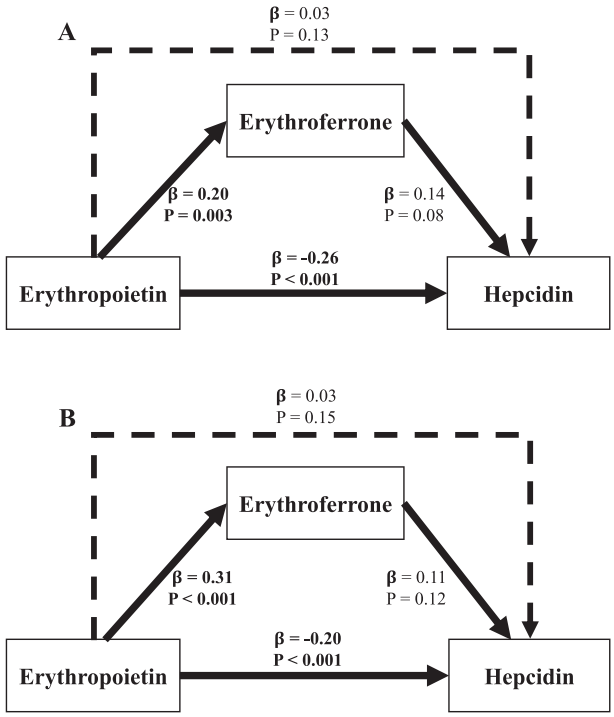

FIGURE 2 Mediation models for erythroferrone, erythropoietin, and hepcidin in pregnant adolescents and women carrying multiple fetuses at midgestation and delivery. Mediation models were utilized to assess direct associations (solid lines) and indirect associations (dashed lines) between these regulatory hormones at midgestation (A) and delivery (B). Bolded coefficients and *P* values indicate significant effects ($P < 0.05$). Hepcidin was measured with different assays in the multiples and adolescent cohorts; *z* scores were created and used for statistical evaluations in the cohort as a whole.

and delivery (Bartlett's $P = 0.003$). This broader range of ERFE concentrations observed in the adolescent cohort at each gestational time point sampled can be visualized by comparing quartiles of ERFE concentrations between cohorts (Table 3). The differences in longitudinal changes in ERFE between cohorts remained after adjusting for differences in maternal race, ppBMI, GWG, parity, or mode of delivery. In the participants with longitudinal measures of ERFE, midgestation ERFE concentrations were significantly positively associated with ERFE concentrations at delivery ($n = 148$; $R^2 = 0.74$, $P < 0.001$).

Regulatory hormone ratios and predictive ability for identifying ID and anemia

Given the known regulatory interrelations between EPO, hepcidin, and ERFE, ratios between these hormones were explored to determine if they would provide additional predictive ability in identifying anemia, IDA, or depleted iron stores than individual hormone measures alone. Additionally, EPO and ERFE may have greater utility in identifying women at an increased risk of ID or anemia than SF or sTfR as they are not impacted by inflammation. At midgestation and delivery, the hepcidin:ERFE ratio explained more variance in EPO ($R^2 = 0.17$, $P < 0.001$; $R^2 = 0.23$, $P < 0.001$, respectively) than hepcidin ($R^2 = 0.10$; $R^2 = 0.09$) or ERFE ($R^2 = 0.10$; $R^2 = 0.15$) alone. The hepcidin:EPO ratio captured the most variance in both SF ($R^2 = 0.20$, $P < 0.001$; $R^2 = 0.24$, $P < 0.001$, respectively) and TBI ($R^2 = 0.22$, $P < 0.001$; $R^2 = 0.30$, $P < 0.001$) compared with either hepcidin:ERFE, ERFE:EPO, EPO, ERFE, or hepcidin.

To further explore the predictive ability of the iron biomarkers evaluated, multiple ROCs were generated, and the AUCs were compared to explore the ability of each iron regulatory hormone and hormone ratio in identifying women with anemia, IDA, or depleted iron stores (Figure 3). At midgestation and delivery, EPO performed best in the combined cohort (midgestation AUC: 0.772; delivery AUC: 0.836), as well as within each individual cohort at identifying anemia and depleted iron stores. The EPO cutoff for anemia at both midgestation and delivery was ~38 mU/mL and the cutoff for depleted iron stores was slightly higher, ~50 mU/mL. Although EPO performed best within each individual cohort, the cutoffs generated to identify women with anemia or depleted iron stores were higher in the multiples cohort than observed in the adolescent cohort. The hepcidin:EPO ratio performed the best at identifying women with IDA at midgestation and delivery and the AUC for hepcidin:EPO was similar to EPO for anemia and IDA.

Discussion

To our knowledge, this is the first study to characterize human ERFE across pregnancy and assess its utility in identifying women at increased risk of anemia, ID, or IDA. Within these 2 pregnant populations, ERFE was found to be significantly positively associated with EPO and sTfR across pregnancy, but despite its known role in hepcidin regulation, no significant correlations were evident between ERFE and hepcidin. Although ERFE was associated with iron status biomarkers, of all hormones evaluated, EPO and the ratio of hepcidin to EPO performed best at identifying pregnant women at increased risk of anemia, ID, or IDA across gestation.

Few data on human ERFE concentrations in serum have been published as a validated human assay only became available in 2017 (35). In our cohort of healthy pregnant women, mean ERFE concentrations were ~0.48 ng/mL at both midgestation and at delivery. Only 2 published studies to date have measured ERFE using the same assay in healthy nonpregnant women. One study reported a median ERFE concentration of 0.32 ng/mL (IQR: 0.01–0.76) ($n = 77$; age 26–60 y) (50). The other study in women (age 18–22 y) reported mean ERFE concentrations of 1.0 ± 1.13 ng/mL in non-ID elite athletes ($n = 35$) and 3.5 ± 5.1 ng/mL ($n = 4$) in elite athletes with SF <12 ng/mL (51). Data on ERFE concentrations in healthy males report slightly higher concentrations (~0.6–1 ng/mL; $n = 80$) (50, 52) than those observed in these pregnant women. In sum, ERFE concentrations in these pregnant women remain substantially lower than values reported among elite male and female athletes (6–11 ng/mL) (51, 53) or individuals with diseases known to induce erythropoietic stress (4, 34, 54–58).

EPO is currently thought to be the main driver of ERFE expression in erythroblasts, and animal studies have shown ERFE functions as the mediating factor between EPO and hepcidin (4, 33, 54). Hepatocyte cell culture studies have shown that ERFE decreases transcription of the hepcidin gene by binding to bone morphogenic protein (BMP) 2/6 (BMP2/6), preventing BMP2/6 binding with the BMP receptor and blocking the downstream signal transduction that activates transcription of the hepcidin gene (59). In our study of pregnant women, no significant correlation between ERFE and hepcidin was observed. Hepcidin is known to be regulated by multiple

TABLE 3 Serum ERFE quartiles in pregnant adolescents and women carrying multiple fetuses[1]

	ERFE, ng/mL			
	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Midgestation				
Multiples	0.13 ± 0.08* (18)	0.34 ± 0.10* (18)	0.70 ± 0.09* (18)	2.78 ± 0.08* (18)
Adolescents	0.04 ± 0.08 (34)	0.23 ± 0.08 (35)	0.57 ± 0.08 (35)	6.22 ± 0.08 (35)
Delivery				
Multiples	0.22 ± 0.08* (12)	0.55 ± 0.07* (13)	1.27 ± 0.08* (12)	4.61 ± 0.08 (12)
Adolescents	0.06 ± 0.07 (45)	0.23 ± 0.06 (47)	0.46 ± 0.06 (47)	4.05 ± 0.07 (46)

[1]Values are means ± SDs (*n*). *Significant difference ($P < 0.05$) between cohorts within each quartile. ERFE, erythroferrone.

competing signals including a pregnancy-related factor, iron status, inflammation, erythropoietic drive, and hypoxia (60–64). Our lack of an association may indicate that these, or other, regulatory signals take precedence over hepcidin regulation during pregnancy, perhaps because ERFE is at most mildly elevated. Additionally, the lack of association between ERFE and hepcidin is similar to findings reported in studies of healthy men and nonpregnant women (50, 53, 55).

Relations between ERFE, EPO, hepcidin, and iron status indicators differed between women carrying multiples and adolescent mothers carrying singletons. The prevalence of anemia was significantly lower among the adolescent mothers

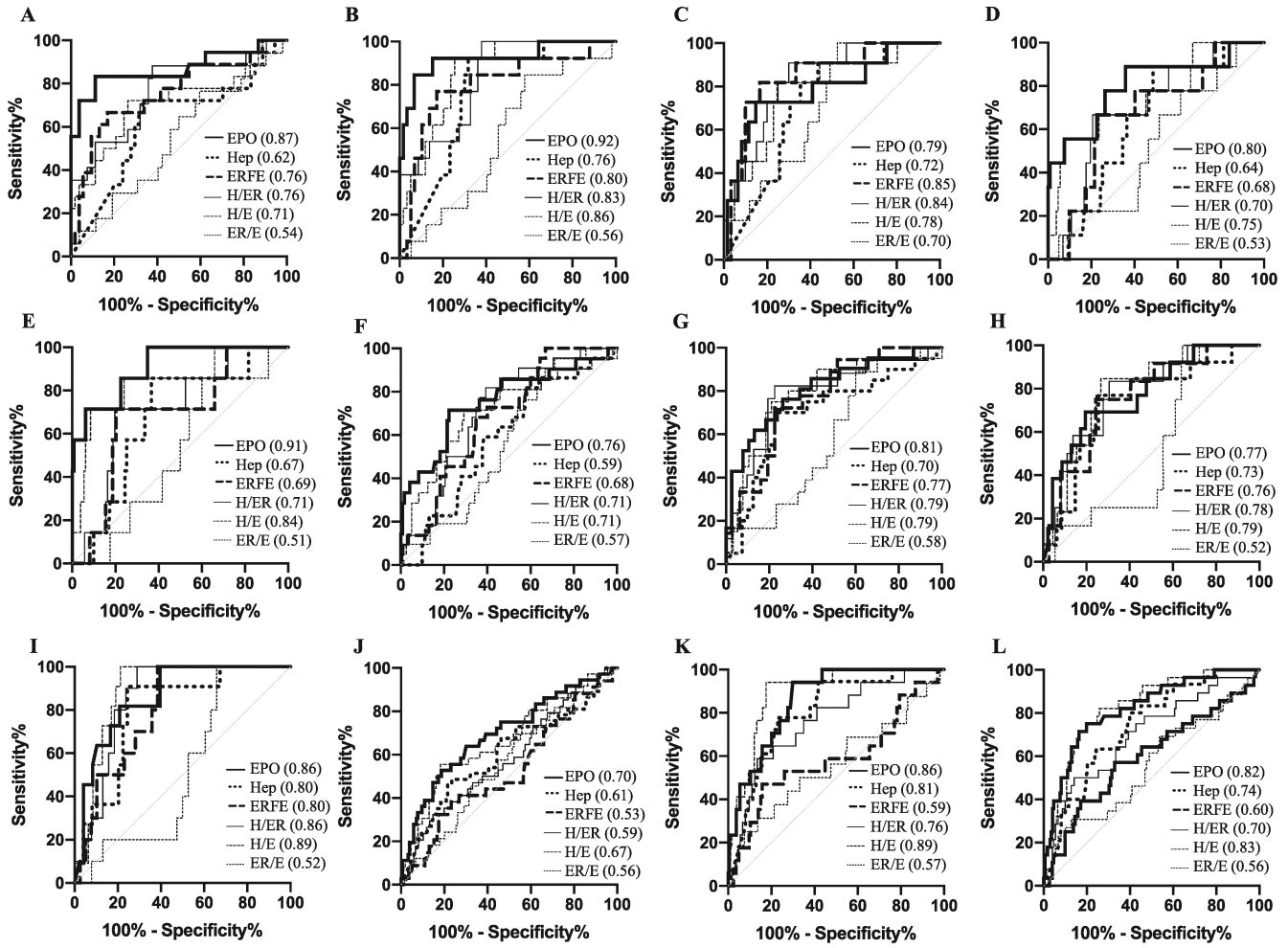

FIGURE 3 ROC and AUC for anemia, iron deficiency anemia and depleted iron stores at midgestation and delivery in women carrying multiple fetuses and pregnant adolescents. ROCs for identifying (A) anemia in women carrying multiple fetuses at mid-gestation, (B) iron deficiency anemia in women carrying multiple fetuses at midgestation, (C) depleted iron stores in women carrying multiple fetuses at midgestation, (D) anemia in pregnant adolescents at midgestation, (E) iron deficiency anemia in pregnant adolescents at midgestation, (F) depleted iron stores in pregnant adolescents at midgestation, (G) anemia in women carrying multiple fetuses at delivery, (H) iron deficiency anemia in women carrying multiple fetuses at delivery, (I) depleted iron stores in women carrying multiple fetuses at delivery, (J) anemia in pregnant adolescents at delivery, (K) iron deficiency anemia in pregnant adolescents at delivery, (L) depleted iron stores in pregnant adolescents at delivery. The value next to the hormone or hormone ratio indicates the AUC. E, EPO, erythropoietin; ERFE, ER, erythroferrone; H, Hep, hepcidin; ROC, receiver operating characteristic curve.

in this study, and ERFE was only weakly associated with EPO in this cohort. The observed differences in predictors of ERFE between cohorts may be due not only to the lower prevalence of anemia in the adolescent cohort but by variables that may differ as a consequence of the biological immaturity of these adolescent mothers and the greater total mass of growing fetuses in the multiples cohort. Studies in adolescent sheep found that biological immaturity was associated with a failure to appropriately expand plasma volume, leading to increased plasma viscosity and greater Hb and plasma protein concentrations (65). In nonpregnant women and men (age 50–80 y), plasma viscosity was found to be inversely associated with EPO production, even when anemia was present (66). In these 2 cohorts, however, there were no significant differences in EPO between groups at midgestation. Additionally, nutrient partitioning between the growing adolescent and developing fetus may differ from that observed among adult pregnant women (67, 68). More data are needed to identify factors responsible for the increased ERFE variability and weaker relation between ERFE and EPO in these adolescent gravidae.

Many studies have published data on hepcidin and EPO across pregnancy, but few have compared the relative utility of these hormones with respect to their ability to identify women with anemia, ID, or IDA. Using an ROC approach, the AUC for hepcidin in identifying women with depleted iron stores was ~0.75 in our study population, a value that is similar to values reported in 3 other studies reporting AUC values of hepcidin for identifying depleted iron stores in pregnant women in developing countries (28, 31, 32). We found that EPO performed better when identifying women with anemia or depleted iron stores (combined cohort: midgestation AUC, 0.77; delivery AUC, 0.84). There are no other published data using an ROC approach to evaluate relations between EPO and iron status in pregnant women. However, other published data have evaluated the predictive ability of erythrocyte protoporphyrin in identifying depleted iron stores in pregnant women and found a slightly lower AUC for this measure (AUC: second trimester, 0.744; third trimester, 0.715) (69). Additionally, in the women carrying multiples, the ROC curve for EPO that was developed to identify the risk of anemia demonstrated a sharp increase in sensitivity while maintaining high specificity at midgestation but not at delivery. This suggests that this indicator may have enhanced diagnostic ability in midpregnancy, a point in pregnancy when interventions to improve iron status can be initiated.

Ratios between iron and erythropoietic regulatory hormones may provide additional predictive ability compared with single-hormone measures. In the current study, the ratio between hepcidin and EPO performed best at identifying women at increased risk for ID or IDA. Although no published data have assessed the utility of hepcidin:EPO, prior data evaluating the predictive ability of the hepcidin to ERFE ratio have been published (70–72). In hemodialysis patients the hepcidin to ERFE ratio was best able to predict increases in Hb concentrations after ferric citrate hydrate administration (71). Another study in thalassemic patients found that the hepcidin to ERFE ratio was significantly lower in thalassemic patients compared with normal controls (70), and Wei et al. (72) found that the ratio between hepcidin and ERFE best predicted the risk of spontaneous abortion.

Our study provides novel data on ERFE in relation to other iron biomarkers, but there are limitations that may impact study findings. Data used in these analyses were obtained from healthy pregnant women, but both pregnant adolescents and women carrying multiples are unique obstetric populations at increased risk of ID and IDA due to the competing demands of continued adolescent growth or multiple fetal/placental units. Additionally, hepcidin was measured using 2 different assays between the cohorts. This makes interpretation of the hormone ratio cutoffs difficult to interpret when comparing between studies.

In conclusion, maternal ERFE was associated with erythropoietic demand during pregnancy, but it was not significantly associated with hepcidin. Relations between ERFE and iron status indicators significantly differed in adolescent pregnancies when compared with adult women carrying multiples and further work is needed to identify factors responsible for the observed differences between populations. In both groups of pregnant women, EPO and the ratio of hepcidin to EPO were best able to identify women at increased risk of anemia, ID, or IDA compared with other iron regulatory hormones and ratios. Of all measures examined, the hepcidin to PO ratio, a ratio that accounts for both ID and erythropoietic drive, was the strongest indicator for identifying women at an increased risk of ID or IDA at term. These findings suggest that ERFE alone is not a sensitive biomarker of iron status or anemia during pregnancy and that EPO and hepcidin are more informative at this time.

Acknowledgments

The authors' responsibilities were as follows—KMD: conducted research, analyzed and interpreted the data, and wrote the manuscript; KOO: designed and conducted research, analyzed and interpreted the data, and wrote the manuscript; RG and EKP: were responsible for the clinical implementation of the studies and assisted with the design of the research and analysis and interpretation of the data and with the preparation of the manuscript. EN and 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.

Data Availability

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

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