Do Extremely Low Gestational Age Neonates Regulate Iron Absorption via Hepcidin?

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Objectives To evaluate whether extremely preterm infants regulate iron status via hepcidin.

Study design In this retrospective analysis of infants from the Preterm Epo Neuroprotection (PENUT) Trial, urine hepcidin (Uhep) normalized to creatinine (Uhep/UCr) was evaluated among infants randomized to erythropoietin (Epo) or placebo.

Results The correlation (*r*) between Uhep/UCr and serum markers of iron status (ferritin and zinc protoporphyrin-to-heme ratio [ZnPP/H]) and iron dose was assessed. A total of 243 urine samples from 76 infants born at 24-27$^{6/7}$ weeks gestation were analyzed. The median Uhep/UCr concentration was 0.3, 1.3, 0.4, and 0.1 ng/mg at baseline, 2 weeks, 4 weeks, and 12 weeks, respectively, in placebo-treated infants. The median Uhep/UCr value in Epo-treated infants were not significantly different, with the exception of the value at the 2-week time point (median Uhep/UCr, 0.1 ng/mg; $P < .001$). A significant association was seen between Uhep/UCr and ferritin at 2 weeks ($r = 0.63$; $P < .001$) and at 4 weeks ($r = 0.41$; $P = .01$) and between Uhep/UCr and ZnPP/H at 2 weeks ($r = -0.49$; $P = .002$).

Conclusions Uhep/UCr values correlate with serum iron markers. Uhep/UCr values vary over time and are affected by treatment with Epo, suggesting that extremely preterm neonates can regulate hepcidin and therefore their iron status. Uhep is suppressed in extremely preterm neonates, particularly those treated with Epo. *(J Pediatr 2022;241:62-7)*.

Optimizing iron status in extremely preterm neonates is important because they are at risk for both iron deficiency and overload.[1-3] Adults regulate their iron status through the action of hepcidin.[4] When individuals are iron-sufficient, hepcidin is up-regulated, leading to decreased iron absorption and availability.[5] Hepcidin also has been shown in adult humans and animal models to be up-regulated in the setting of inflammation.[6,7] presumably to sequester iron in the setting of potential siderophilic bacterial infection.

Whether extremely preterm neonates are able to regulate iron intake through hepcidin titration is not known. Lorenz et al showed a correlation between hepcidin levels derived from cord blood and ferritin values; however, the persistence of this relationship over time was not evaluated.[8] Similar to adults, hepcidin and pro-hepcidin levels in term and preterm infants vary in response to inflammation, specifically infection and red blood cell transfusion.[9-11] This suggests that infants may be able to regulate their hepcidin level.

Müller et al showed a significant positive correlation between urine hepcidin (Uhep) and serum hepcidin, which allows noninvasive measurement of hepcidin levels.[12] We hypothesized that extremely preterm neonates would be able to adjust hepcidin levels in response to iron status. Specifically, we aimed to address the following questions: (1) does hepcidin correlate with other markers of iron status (ferritin and zinc protoporphyrin-to-heme ratio [ZnPP/H]) and iron dose?; (2) how does hepcidin vary over time in extremely preterm neonates?; and (3) how is this relationship impacted by exogenous erythropoietin (Epo) treatment?

Methods

We conducted a retrospective analysis of urine samples that had been collected prospectively at the University of Washington as part of the Preterm Epo for

Epo	Erythropoietin
IS	Internal standard
IV	Intravenous
PAR	Peak area ratio
PENUT	Preterm Epo Neuroprotection Trial
Uhep	Urine hepcidin
Uhep/UCr	Urine hepcidin-to-creatinine ratio
ZnPP/H	Zinc protoporphyrin-to-heme ratio

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Neuroprotection Trial (PENUT; U01NS077953). The PENUT Trial protocol and primary outcome were published previously.[13,14] In brief, PENUT was a prospective, multicenter, randomized, placebo-controlled trial aimed at evaluating the potential neuroprotective effects of Epo. Sequential urine samples collected as part of the study were available for analysis. Parents of study participants provided written informed consent for participation in the PENUT Trial, for collection of urine samples, and future analysis. Our ancillary study was deemed exempt by the Institutional Review Board at the University of Washington based on the use of deidentified patient data and was approved by the PENUT Ancillary Study Committee.

All infants who had sufficient urine specimens available for analysis at 4 predetermined time points were included in this study. Measurements were obtained from urine samples collected early (baseline, day of life 0-7 days) and at 2 weeks (±7 days), 4 weeks (±7 days), and 12 weeks of life (or last available urine).

Demographic characteristics as well as information regarding clinical care of participants, such as red blood cell transfusions, were collected from the deidentified PENUT database.

Iron status was assessed by ferritin and ZnPP/H, collected as part of routine neonatal care. These values were abstracted from the PENUT Database. Iron supplementation guidelines were in place at the study institution during the period of sample collection. The guidelines recommend initiating iron supplementation at 2-4 mg/kg/day beginning at 1 week of age. Ferritin and ZnPP/H were measured every 2-4 weeks, and iron dose was adjusted based on these values, up to a maximum of 12 mg/kg/day.

The ZnPP/H was determined using a ProtoFluor-Z hematofluorometer (Helena Laboratories). Cells were centrifuged to remove possible interference, then suspended in 0.9% saline containing 2.5% bovine serum albumin before the measurement of ZnPP/H. The analytical within-day coefficient of variation is 4.31% in the normal range and 2.94% in the high range.

Hepcidin was measured by high-performance liquid chromatography-mass spectrometry using a validated methodology published by Lefebvre et al.[15] In brief, samples were measured by isolating parent mass-to-charge ions of 698.1 and 558.9 for hepcidin (doubly and triply charged state, respectively), and 703.1 for the C13/N15-labeled internal standard (IS). The fragment ion for both analytes and IS monitored was mass-to-charge 341. Chromatographic separation was achieved using a slower gradient than that reported by Lefebvre et al to remove interference peaks. Urine creatinine (UCr) analysis was also done by high-performance liquid chromatography using mock urine to generate calibration curves.

Data were analyzed using QuanLynxs software (Waters). A linear equation was generated by analyzing known concentrations of hepcidin (0.5-250 ng/mL) and creating a peak area ratio (PAR) of the analyte of interest over the IS. The resulting PARs were then plotted against the concentrations to generate slope and intercepts. This equation was then applied to the PARs for the calibration curve to generate values and determine acceptability. The acceptability of quantitation level was determined by a variance <15% from the expected return value.

Statistical Analyses

Ferritin and ZnPP/H measurements captured clinically within ±3 days of urine collection were included in the analyses. The mean enteral iron dosage at weeks 2, 4, and 12 (±3 days) was calculated and standardized by the average weight (kg) over the same time period. Uhep, Uhep/UCr, serum ferritin, and serum ZnPP/H measurements were compared by randomized treatment group using separate Wilcoxon rank-sum tests at weeks 0, 2, 4, and 12. Median and IQR were presented for each measurement over time by treatment group. Week 2 Uhep/UCr were similarly summarized by maternal and neonatal demographic and clinical characteristics. Associations between Uhep/UCr and ferritin, ZnPP/H, and average enteral iron intake measurements at weeks 2, 4, and 12 were evaluated separately using the Spearman correlation coefficient. Mean transfusion volume in infants in the Epo- and placebo-treated groups were recorded and compared but were not included in this analysis. All statistical analyses were performed using R version 4.0.2,[16] and statistical significance was declared using a 2-sided type 1 error rate of 0.05; no correction for multiple testing was used.

Results

A total of 243 samples derived from 76 infants had sufficient urine for analysis at the prespecified time points along with median Uhep/UCr values at the 2-week time point (**Table**). Uhep/UCr values at 2 weeks were higher in males and generally decreased with gestational age, consistent with increasing iron stores over gestation. Uhep/UCr values also were higher in infants who had packed red blood cell transfusions. No other demographic characteristics were associated with significant differences.

Figure 1 displays the correlations between Uhep/UCr and 2 serum markers of iron status, ferritin, and ZnPP/H. **Figure 1**, A shows a significant positive association between Uhep/UCr and serum ferritin at 2 and 4 weeks with a Spearman correlation coefficient of $r = 0.63$ ($P < .001$) and $r = 0.41$ ($P = .01$), respectively. A negative association between Uhep/UCr and serum ZnPP/H was observed at 2 weeks ($r = -0.49$; $P = .002$), although the association did not persist at the 4-week time point (**Figure 1**, B).

Figure 2 shows the change in iron measures and Uhep over time, stratified by treatment arm. In terms of iron status, **Figure 2**, A and B show that ferritin values were lower and ZnPP/H values higher in Epo-treated infants throughout all time points assessed, consistent with Epo-treated infants being more iron deficient. The difference in ferritin and ZnPP/H across groups lessens by 12 weeks of age when Epo treatment was completed in the Epo group, and both groups have lower ferritin measures.

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Table. Demographic characteristics of all infants with at least 1 hepcidin measurement

Characteristics	Overall cohort summary	Week 2 Uhep/UCr, ng/mg, n, median (IQR)
Number of infants	76	59, 0.32 (0.05-1.45)
Maternal characteristics		
Maternal age, y, mean (SD)	30.1 (6.6)	
Maternal age, y, n (%)		
<30	34 (45)	29, 0.33 (0.05-2.29)
≥30	42 (55)	30, 0.22 (0.05-1.41)
Maternal education, n (%)		
Hight school or less	20 (28)	15, 0.41 (0.05-1.58)
Some college	32 (44)	27, 0.15 (0.05-0.81)
Bachelors degree or greater	20 (28)	14, 0.41 (0.09-1.44)
Pregnancy-induced hypertension, n (%)		
Yes	17 (22)	12, 0.12 (0.04-0.60)
No	59 (78)	47, 0.41 (0.06-1.80)
Maternal obesity, n (%)		
Yes	17 (22)	11, 0.56 (0.09-1.15)
No	59 (78)	48, 0.27 (0.05-1.64)
Maternal smoking, n (%)		
Yes	5 (7)	3, 0.19 (0.12-0.30)
No	71 (93)	56, 0.34 (0.05, 1.61)
Gestational diabetes, n (%)		
Yes	4 (5)	4, 0.19 (0.12-0.30)
No	72 (95)	55, 0.34 (0.05-1.61)
Delayed cord clamping, n (%)		
Yes	47 (62)	37, 0.22 (0.05-0.89)
No	29 (38)	22, 0.58 (0.05-2.27)
Neonatal characteristics		
Sex, n (%)		
Male	38 (50)	**31, 0.55 (0.11-3.23)**
Female	38 (50)	**28, 0.12 (0.05-0.89)**
Multiple gestation, n (%)		
Yes	20 (26)	18, 0.33 (0.09-1.35)
No	56 (74)	41, 0.31 (0.05-1.48)
Gestational age at birth, wk, mean (SD)	26.2 (1.1)	
Gestational age, n (%)		
24 wk	13 (17)	**10, 1.38 (0.46-3.22)**
25 wk	15 (20)	**13, 0.63 (0.14-1.75)**
26 wk	24 (32)	**16, 0.10 (0.05-0.43)**
27 wk	24 (32)	**20, 0.13 (0.04-0.75)**
Birth weight, g, mean (SD)	797 (176)	
Birth weight, g, n (%)		
<780	38 (50)	26, 0.60 (0.06-1.46)
≥780	38 (50)	33, 0.22 (0.05-0.74)
SGA, n (%)		
Yes	5 (7)	2, 0.08 (0.06-0.10)
No	71 (93)	5, 0.33 (0.05-1.47)
Received PRBC transfusion by day 14, n		
Yes	41	**31, 0.90 (0.07-2.93)**
No	35	**28, 0.20 (0.04-0.58)**

PRBC, packed red blood cell; *SGA*, small for gestational age.
Descriptive statistics of the total study cohort. The rightmost column shows the number of infants who had 2-week Uhep values available for analysis, as well as the median and IQR of Uhep/UCr values. Week 2 Uhep/UCr measurements differed between levels of variables in bold type (sex, $P = .02$; gestational age, $P = .04$; PRBC transfusion at any time. $P = .001$; PRBC transfusion by day 14, $P = .03$).

Figure 2, C and D shows that Uhep and Uhep/UCr values varied over time in Epo-treated infants. In contrast, placebo-treated infants had lower Uhep and Uhep/UCr values at all time points. Of note, the Uhep and Uhep/UCr values at 2 weeks of life were significantly different between the Epo-treated and placebo-treated infants, concordant with the time of greatest difference in iron status between the groups based on ferritin values. To further evaluate this time point, the associations between Uhep/UCr at 2 weeks and serum iron levels at 2 weeks stratified by treatment group are shown in Figure 3. The relationship between Uhep/UCr and serum iron values at 2 weeks was similar in Epo-treated and placebo-treated infants, although the hepcidin values differed in the 2 groups.

To evaluate the relationship between iron dose and Uhep values, associations between average enteral iron dose and intravenous (IV) iron dose were examined and are summarized in Figure 4, A and B, respectively (available at www.jpeds.com). As anticipated in this retrospective study, these data suggest that clinicians responded to laboratories values that indicated iron deficiency by increasing iron doses. Thus, infants with low iron status (and thus low hepcidin levels) received higher doses of enteral iron supplements. Consequently, increasing average daily enteral iron intake was associated with a reduced Uhep/UCr at both week 2 ($r = -0.47$; $P < .001$) and week 4 ($r = -0.30$; $P = .03$) (Figure 4, A). By week 2 and week 4, the majority of infants were no longer given IV iron (Figure 4, B); however, those who continued to receive IV iron at week 2 had an increased Uhep/UCr ($r = 0.32$; $P = .02$). Owing to the low number of paired Uhep/UCr and ferritin, ZnPP/H, enteral, and IV iron dose values available at the 12-week time point, 12-week data are not included in this figure. To assess whether the relationship between iron dose and Uhep values differed in Epo-treated and placebo-treated infants, the association between Uhep or Uhep/UCr and iron dose at 2 weeks of life was evaluated (Figure 5; available at www.jpeds.com). These associations were similar in the Epo-treated and placebo-treated infants.

Transfusion status in the study infants was descriptively reviewed. The mean transfusion volume in this study cohort was 38.1 ± 45.8 mL overall, with a higher mean transfusion volume received by infants in the placebo group compared with the Epo group (48.0 mL vs 30.2 mL). The majority of transfusions were given early in life, with the median time of first transfusion of 8 days (IQR, 3-14 days).

Discussion

In this analysis of extremely preterm infants enrolled in the PENUT trial, we evaluated the association between Uhep value and other markers of iron deficiency, trends in Uhep values over time, and the impact of Epo on Uhep. As hypothesized, we found a significant association between Uhep/UCr value and markers of iron status, with a positive association seen with ferritin and a negative association seen with ZnPP/H at the 2- and 4-week time points. The relationship between Uhep/UCr and iron markers was most robust at the 2-week time point, owing in part to more data points available for analysis and thus added power at this time point. Similar findings have been reported by Müller et al, who evaluated Uhep and serum hepcidin values in a small cohort of preterm infants and found significant correlations between hepcidin values and markers of iron sufficiency, using

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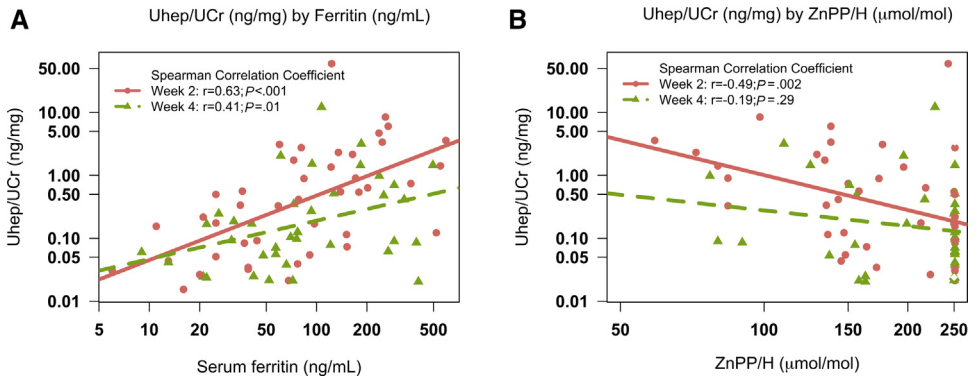
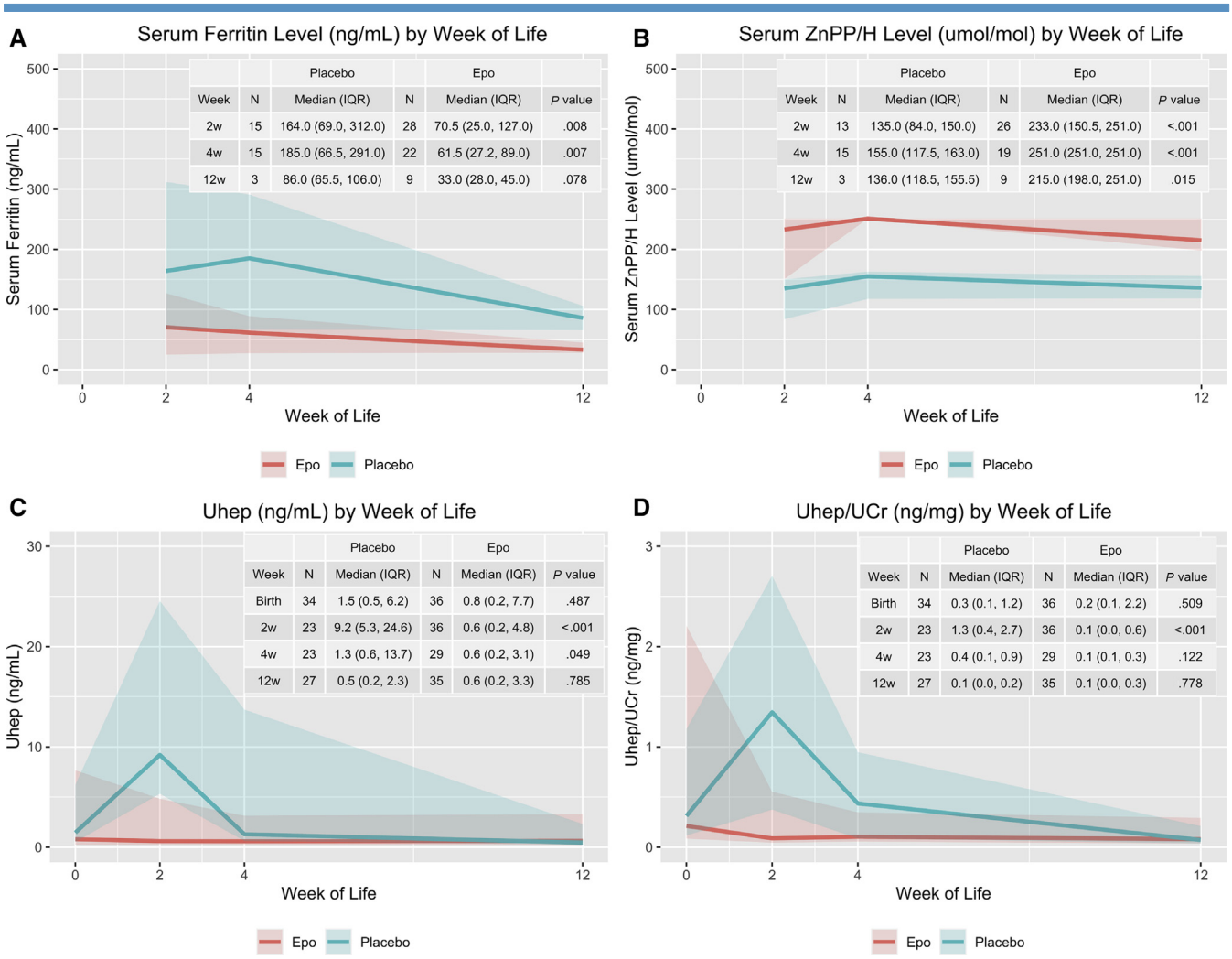


Figure 1. Scatterplot of Uhep/UCr and iron markers at 2 and 4 weeks of life. The associations between **A,** Uhep/UCr and ferritin and **B,** ZnPP/H were evaluated at 2 and 4 weeks of age. Spearman correlation coefficients for these associations are summarized (*inset*).



A		Placebo		Epo	
Week	N	Median (IQR)	N	Median (IQR)	*P* value
2w	15	164.0 (69.0, 312.0)	28	70.5 (25.0, 127.0)	.008
4w	15	185.0 (66.5, 291.0)	22	61.5 (27.2, 89.0)	.007
12w	3	86.0 (65.5, 106.0)	9	33.0 (28.0, 45.0)	.078

B		Placebo		Epo	
Week	N	Median (IQR)	N	Median (IQR)	*P* value
2w	13	135.0 (84.0, 150.0)	26	233.0 (150.5, 251.0)	<.001
4w	15	155.0 (117.5, 163.0)	19	251.0 (251.0, 251.0)	<.001
12w	3	136.0 (118.5, 155.5)	9	215.0 (198.0, 251.0)	.015

C		Placebo		Epo	
Week	N	Median (IQR)	N	Median (IQR)	*P* value
Birth	34	1.5 (0.5, 6.2)	36	0.8 (0.2, 7.7)	.487
2w	23	9.2 (5.3, 24.6)	36	0.6 (0.2, 4.8)	<.001
4w	23	1.3 (0.6, 13.7)	29	0.6 (0.2, 3.1)	.049
12w	27	0.5 (0.2, 2.3)	35	0.6 (0.2, 3.3)	.785

D		Placebo		Epo	
Week	N	Median (IQR)	N	Median (IQR)	*P* value
Birth	34	0.3 (0.1, 1.2)	36	0.2 (0.1, 2.2)	.509
2w	23	1.3 (0.4, 2.7)	36	0.1 (0.0, 0.6)	<.001
4w	23	0.4 (0.1, 0.9)	29	0.1 (0.1, 0.3)	.122
12w	27	0.1 (0.0, 0.2)	35	0.1 (0.0, 0.3)	.778

Figure 2. Uhep levels and measures of iron status in Epo-treated and placebo-treated infants over time. Median **A,** serum ferritin; **B,** serum ZnPP/H; **C,** Uhep; and **D,** Uhep/UCr values at 2, 4, and 12 weeks of life are shown for Epo-treated and placebo-treated infants. IQRs are depicted by shading. The comparisons of median values at 2, 4, and 12 weeks of age in placebo-treated vs Epo-treated infants are summarized by *P* values (*inset*).

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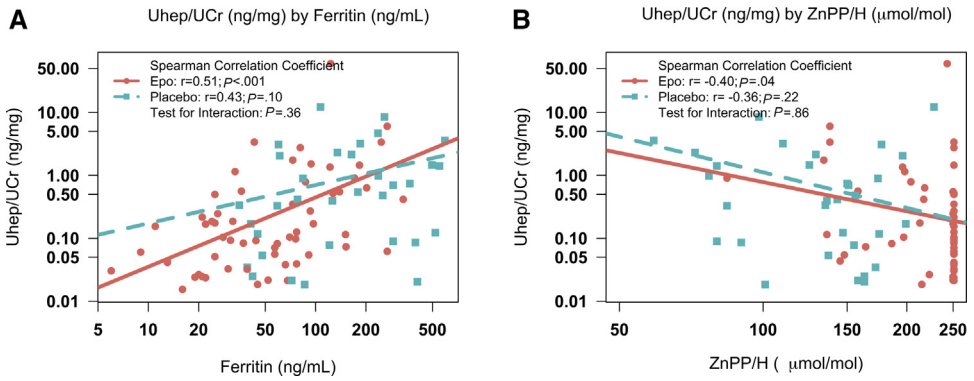

Figure 3. Correlation between Uhep/UCr and iron measurements, stratified by treatment arm, at 2 weeks of life. The associations between **A,** Uhep/UCr and ferritin and **B,** ZnPP/H were evaluated at 2 weeks of age in Epo-treated and placebo-treated infants. The correlation between Epo-treated and placebo-treated subjects at 2 weeks is summarized by Spearman correlation coefficients (*inset*).

ferritin, reticulocyte hemoglobin content, and soluble transferrin receptor/ferritin ratio.[12] Stripeli et al also showed a positive association between hepcidin and total iron-binding capacity and transferrin in a cohort of 19 preterm infants.[17] In the present study, we built on these findings by examining the relationship in a larger population over multiple time points and by evaluating the effect of Epo treatment on Uhep. Our findings, and those of previous studies, support a correlation between hepcidin values and iron status in neonates, further supporting the hypothesis that preterm infants are able to regulate their iron status through hepcidin. Moreover, the association between Uhep and serum iron markers suggests the possibility of using a noninvasive urinary marker to help monitor the iron status of neonates. Further studies are needed to establish target Uhep values in extremely preterm neonates and to evaluate whether these values correlate with long-term outcomes.

We found that Uhep values vary over time, and that this variation is dependent on Epo treatment. Epo-treated infants had low Uhep values at all time points studied. In contrast, placebo-treated infants had higher mean Uhep values, particularly at the 2-week time point. In older children and adults, hepcidin values increase as iron sufficiency improves. We surmise that the lower Uhep values in Epo-treated infants reflects that these infants are responding appropriately to the increased iron debt caused by up-regulation of Epo-stimulated erythropoiesis.

With the exception of the 2-week time point in the placebo-treated infants, Uhep values were low in both groups and decreased over time in the placebo-treated group. This suggests an unmet iron debt in extremely preterm neonates despite an average transfusion volume of 48.0 mL in placebo-treated infants and 30.2 mL in Epo-treated infants,[14] plus enteral iron supplementation up to 12 mg/kg/day given in the PENUT Trial, a dose considerably higher than the supplementation guidelines recommended by the American Academy of Pediatrics.[18] This suggests the recommended iron supplementation may be insufficient to meet the demands of extremely preterm infants or that alternative sources of iron, such as IV formulations, may be needed to achieve sufficiency, particularly in infants treated with erythropoietic-stimulating agents. The advent of third-generation slow-release IV iron formulations may provide an attractive new therapeutic approach to maintaining iron sufficiency in extremely preterm infants who remain iron-deficient despite oral supplementation.

In this retrospective analysis, we found a negative association between enteral iron dose and Uhep levels at 2 and 4 weeks, suggesting that clinicians are appropriately responding to iron deficiency by increasing iron dose in the setting of low iron status (which correlates with low hepcidin values). Conversely, there was a positive association between IV iron dose and Uhep at 2 weeks, which may suggest that infants are more responsive to IV iron (which would imply that IV iron supplementation may be preferable) or that infants who received IV iron in this study were more likely to have inflammatory conditions, which are associated with increased hepcidin values.[9,12] Given the known association between hepcidin and inflammation, we posit that the positive association between IV iron dose and Uhep in this study reflects a greater degree of illness and inflammation in those infants treated with IV iron rather than improved iron status. This is likely because infants were given enteral iron unless made nil per os due to illness.

The primary limitation of this study is that it is a secondary analysis of prospectively collected data and thus the direction of causality between relationships cannot be ascertained, which is particularly relevant to iron supplementation data. Based on PENUT Trial guidelines, clinicians were advised to increase iron supplementation doses based on ferritin and ZnPP/H values. Thus, those infants who received higher iron supplementation doses were likely those who were most iron-deficient at the time of ferritin determination. This leads us to hypothesize that negative association seen between Uhep and iron supplementation values reflects that those infants with high iron supplementation were the most

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iron-deficient (and thus had suppressed Uhep values), but this cannot be determined based on our data. This hypothesis is supported by prospective research by Berglund et al, who showed a positive association between hepcidin and iron dose in a cohort of low birth weight infants.[19]

Transfusion of red blood cells provides an iron source and also increases circulating inflammatory cytokines acutely,[20] both of which can affect hepcidin. Iron is released and recycled as the transfused cells break down. Widness et al reported a mean lifespan of transfused adult red blood cells in a preterm infant of 56.4 ± 7 days (range, 46-68 days), with an estimated half-life of approximately 30 days.[21] In our study, the median postnatal time of first transfusion was day 8 (IQR, days 3-14). Our placebo-treated infants received a mean transfusion volume of 48 mL, compared with 30.2 mL in Epo-treated infants, an 18-mL difference. Based on the assumption that 1 g of hemoglobin contains 3.47 mg of iron, and the hemoglobin value of packed red blood cells is approximately 25 gm/dL (hematocrit, 75%), the difference in iron load of the placebo-treated infants compared with the Epo-treated infants was 13 mg, a difference that is unlikely to have affected the 2-week hepcidin measurement, given the timing of transfusions and the half-life of transfused blood. We recognize, however, that not including this iron source in our analysis underestimates the iron supplementation in infants, particularly in those who received treatment with placebo. Thus, the higher Uhep/UCr values seen at the 2-week time point in the placebo-treated infants may reflect the inflammation associated with transfusion, or an increased iron load.

Uhep values correlate with ferritin, ZnPP/H, and possibly supplemental iron. Furthermore, Uhep values vary over time in extremely preterm neonates and are modulated by Epo treatment. Our results suggest that infants have an intact erythropoietin-erythroferrone-hepcidin-iron axis and thus are able to regulate their iron status. Having an intact iron regulation system is critical to ensuring the safety of iron administration in this population. The correlation between Uhep and serum iron markers suggests an opportunity to develop noninvasive methods of assessing iron status in extremely preterm neonates. ■

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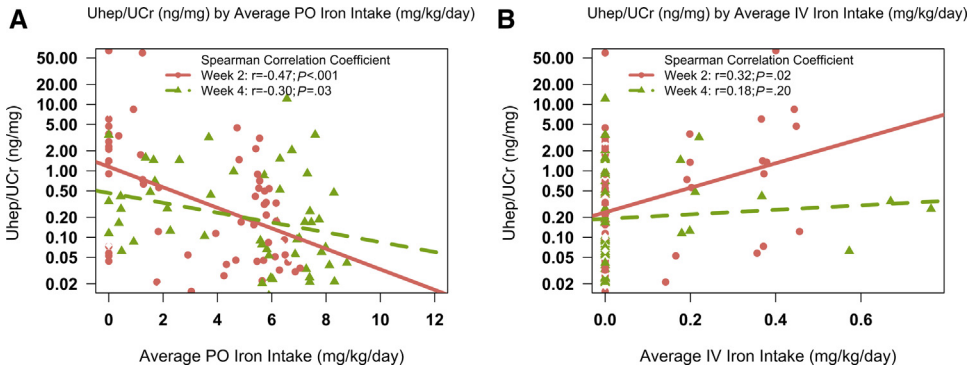

Figure 4. Scatterplot of Uhep/UCr and iron dose at 2 and 4 weeks of life. The associations between Uhep/UCr and **A,** average enteral iron intake and **B,** average IV iron intake were evaluated at 2 and 4 weeks of age. Spearman correlation coefficients for these associations are presented (*inset*). *PO*, by mouth.

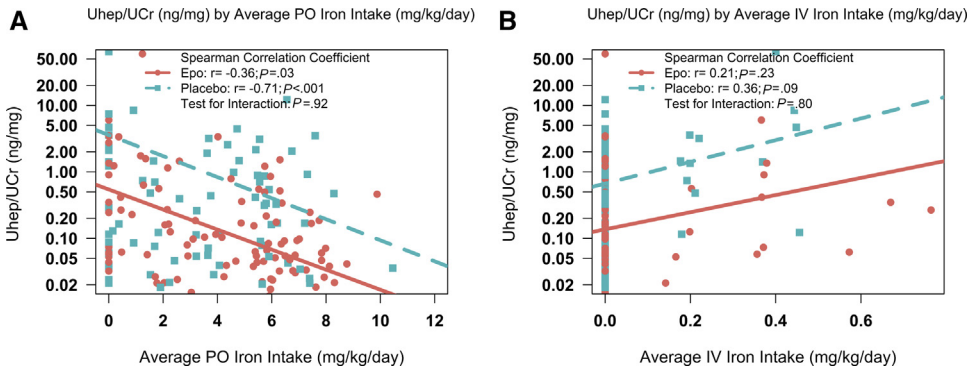

Figure 5. Correlation between Uhep/UCr and iron dose, stratified by treatment arm, at 2 weeks of life. The associations between Uhep/UCr and **A,** average enteral iron intake and **B,** average IV iron intake were evaluated at 2 weeks of age in Epo-treated and placebo-treated infants. The correlation between Epo-treated and placebo-treated subjects at 2 weeks is summarized by Spearman correlation coefficients (*inset*). *PO*, by mouth.

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