Reticulocyte Enrichment of Zinc Protoporphyrin/Heme Discriminates Impaired Iron Supply During Early Development

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ABSTRACT: In infants and children, elevated whole blood zinc protoporphyrin/heme (ZnPP/H) measures iron-deficient (ID) erythropoiesis. Because immature erythrocytes are less dense than mature erythrocytes, we hypothesized that the sensitivity of ZnPP/H is improved if measured in the least dense cells. Blood was collected from control suckling, mildly and severely ID suckling rats. Cord blood was collected after uncomplicated pregnancies (control), diabetic pregnancies (severe ID) and after pregnancies at-risk for iron deficiency (mild ID). ZnPP/H was measured before and after a two-step density centrifugation to obtain the lightest 6.25% of erythrocyte (top fraction). The difference between whole blood and top fraction was defined as ΔZnPP/H. In rats, although the whole or top ZnPP/H differed by postnatal age, ΔZnPP/H was greatest after the interval with least body iron accrual. In either rats or humans with mild ID, whole blood ZnPP/H was similar to, but ΔZnPP/H was greater than controls. In rats and newborn humans, ΔZnPP/H is more sensitive than whole blood ZnPP/H in identifying conditions associated with impaired erythrocyte iron delivery and may become a useful tool in measuring erythrocyte iron incorporation in early development. (*Pediatr Res* **64:** 63–67, 2008)

There is need for a sensitive clinical index of iron-deficient (ID) erythropoiesis in growing premature infants. In premature infants, clinical practices, such as conservative transfusion criteria and erythropoietin therapy may deplete tenuous iron stores (1–4). When iron supply is limited, zinc replaces iron in the protoporphyrin IX ring to form zinc protoporphyrin (ZnPP) (5). An increased ratio of whole blood ZnPP-to-heme (ZnPP/H) reflects impaired erythrocyte iron incorporation (5,6). In older infants, whole blood zinc protoporphyrin/heme (ZnPP/H) is a more sensitive indicator of early, preanemic iron deficiency than Hb or ferritin (7). Whole blood ZnPP/H from cord blood or ill newborns also reflects iron status (4,8). ZnPP/H is higher in newborns born small for dates and offspring of mothers with insulin-treated diabetes (IDM), two conditions commonly exhibiting severely impaired tissue iron content, *i.e.*, severe iron deficiency (severe ID) at birth (4,8). In premature infants, whole blood ZnPP/H falls in response to iron therapy (9) or with indirect delivery of iron *via* transfu-

sion (3). In premature infants, whole blood ratios are inversely related to gestation (8), a clinical disadvantage because of gestation-based normal values are necessary to guide interpretation (4,8–10).

In mild ID, when Hb levels have not yet fallen, employing a more sensitive test of iron incorporation would be useful to facilitate earlier treatment. When iron demands are great and iron is limited, testing the iron status of reticulocytes, the most recently produced erythrocytes, would decrease the number of false negative assessments and improve the sensitivity of ZnPP/H to detect impaired iron incorporation. Previous work supports that measuring iron incorporation into reticulocytes (reticulocyte Hb, or percent hypochromic reticulocytes) with clinical flow cytometry-based automated hematology analyzers is able to identify mild ID (1,8,11–15). Although powerful, sophisticated instrumentation to investigate these parameters is not universally available. However, immature erythrocytes can also be separated based on density centrifugation. After centrifugation, the lighter (top) fraction contains greater relative numbers of reticulocytes than is seen in whole blood. Employing this low tech approach, we sought to examine whether reticulocyte enrichment *via* differential centrifugation (16) would allow us to examine the clinical utility of the difference between whole blood and top fraction ZnPP/H, *i.e.*, the ΔZnPP/H. Because rats are used to model erythropoiesis, are more immature than term humans, and use iron for rapid growth and exhibit differences in ZnPP/H based on iron status (17), the rat model may have utility in assessing whole blood, top fraction, and ΔZnPP/H. Compared with humans, the rats exhibit an exaggerated reticulocytosis, but this characteristic can be exploited by shortening the duration of experimentally-induced mild ID necessary to assess a response. Because whole blood ZnPP/H has been shown to respond to iron treatment in humans (9), it is feasible that whole blood, top fraction, and ΔZnPP/H would also be responsive to iron treatment in newborn rats.

We hypothesized that ZnPP/H measured in the least dense erythrocytes is reproducible and that ΔZnPP/H is more sensitive in detecting mild ID than whole blood ZnPP/H during early rat and human development.

Abbreviations: IDM, offspring of diabetes; **mild ID,** mild iron deficiency; **mild ID+Fe,** mild ID with oral iron; **severe ID,** severe iron deficiency anemia; **ZnPP/H,** zinc protoporphyrin/heme

MATERIALS AND METHODS

Animals. Sprague-Dawley dams (Charles River Laboratories, Wilmington, MA) and pups were studied. UW Animal Care and Use Committee approved the study. Dams were fed Harlan Teklad basic rodent diet, #8604, with 325 mg/kg iron in the diet. Control pups with litter size of 10 defined normal developmental ZnPP/H ratios. In the developmental studies, rats were examined at postnatal day (P): P1 newborn, P4 suckling, P8 suckling, P12 suckling, P18 weanling and adults (10–15 wk).

Control pups were used to develop normative values for ZnPP/H. For treatment studies, a subset of rats underwent randomization to dam-fed (but handled daily) or experimental manipulation with an artificial cow milk-based formula with insufficient iron to meet the needs of suckling rats, 4 mg/L elemental iron using ferrous sulfate. Formula-fed rats were removed from the dam and artificially fed by gastrostomy in temperature-controlled pint-sized polypropylene deli cups as previously described (17,18). Feeds were adjusted to maintain daily weight gain equal to dam-fed rats with a litter size of 10. Artificial feeding began at P4 for severe ID with anemia or P8 for mild ID without anemia. An additional group, mild ID+Fe, included artificial feeding beginning at P8 supplemented with a once daily dose of 6 mg·kg^{-1} oral ferrous sulfate (Fer-In-Sol, Mead Johnson, Evansville, IN). Formula groups were compared with dam-fed control and killed at P12.

Blood analysis and body iron determination. Rats from both developmental and treatment groups were randomly selected for either blood analysis or body iron determination. In the blood group, after lethal isofluorane anesthesia, blood was drawn *via* cardiac puncture and placed into EDTA. For rats assigned to body iron determination, no blood was drawn. Midline incision was performed and gastrointestinal tract was removed from the body to remove iron not yet absorbed, but residing within the gastrointestinal lumen. Blood lost during the gastrointestinal harvest was included with the body. The body without gastrointestinal tract was analyzed for iron content by atomic absorption after nitric acid/perchloric acid digestion. Briefly, the bodies were predigested for 24–48 h at RT in 30 mL concentrated nitric acid and then transferred to Teflon digest tubes with an additional 10 mL of concentrated perchloric acid. The samples were digested at 90°C for 1.5 h, then 200°C until digests were clear. Total Fe concentration of the digests was measured, with accompanying ferrous nitrate reference standards on a Perkin Elmer 2280 Atomic Absorption Spectrophotometer (Waltham, MA).

Human participants. Cord blood samples from four separate groups of newborns were analyzed. EDTA-anticoagulated cord blood is normally collected at all deliveries in the Birthing Center at Meriter Hospital, Madison, WI and held for 7 d in the Blood Bank. We obtained the blood just before samples were to be discarded. Blood was obtained between June 2005 and December 2006. The term control group was randomly collected from normal term deliveries. The second group, severe ID, was identified by Nursery Intensive Care Unit (NICU) admission, from term/near-term deliveries with IDM during pregnancy, because of the marked risk for abnormal iron status in IDMs severely affected by maternal disease (4,8). The third, mild ID group was a subset of larger that was identified for being at-risk for maternal iron deficiency. From the larger at-risk group, we identified a subset with normal cord whole blood ZnPP/H (10), but cord plasma ferritin values in the lowest quartile in our lab, and called this group the mild ID group. The fourth, premature group included cord blood from newborns with birth weight less than 1500 g, but without fetal conditions known to impair iron status, such as maternal diabetes or growth restriction. Because cord ZnPP/H ratios in premature normally fall as gestation increases (4,8), this group was included to determine whether ΔZnPP/H is observed in premature newborns. The premature and IDM groups were identified from the Meriter Hospital NICU admission log and the at-risk group from the healthy newborn admission log. No other data were collected from these mother–child dyads. Approvals from the UW-Madison Human Subjects Committee and Meriter Hospital Institutional Review Board were obtained to analyze deidentified samples.

Laboratory methods. ZnPP/H was determined before (whole blood) and after (top) the density centrifugation step. Samples were tested for clots, stored at 4°C, and hematology profile performed by a Coulter MD Coulter Counter, (Coulter-Beckman, Hialeah, FL). An aliquot of whole blood was centrifuged in a 500 μL microcentrifuge tube (Fisher Scientific, Hampton, NH) at 800*g* in an Eppendorf, 5415D Microcentrifuge (Eppendorf AG, Hamburg, Germany) for 4 min. Plasma was removed. The cell pellet was rinsed in PBS three times to remove interfering pigments (19). After rinsing, packed red blood cells were diluted 1:1 with PBS and whole blood ZnPP/H determined with a front-face hematofluorometer (Aviv Biomedical, Lakewood, NJ).

To obtain ZnPP/H from the top fraction, or least dense, immature erythrocytes, we used the principle of density gradient centrifugation (11,16,20). We centrifuged an aliquot of whole blood for 30 min at 1500*g* (16) in a 400 μL 8 × 48 mm microcentrifuge tube (Fisher Scientific, Waltham, MA) in an
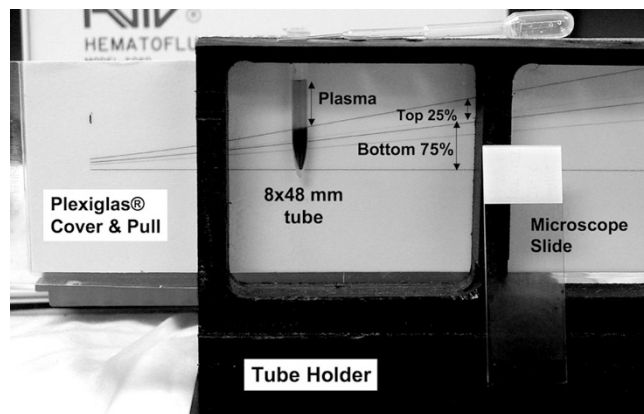
Figure 1. Polyethylene tube reader and sample. The 8 × 48 mm microhematocrit tube is shown with arrows indicating plasma, top 25% of packed cells and bottom 75% of packed cells. The Plexiglas cover and pull are indicated. A microscope slide is included to show size.

Eppendorf, 5415D Microcentrifuge. Plasma was removed with a Fisherbrand gel loading pipet tip (Fisher Scientific) and set aside. The top 25% of packed cells was identified using a custom-made polyethylene Tube Reader (Fig. 1). The top 25% of cells were transferred to a new 8 × 48 mm tube, an equal volume of plasma added, and centrifugation repeated. The top 25% of these cells were transferred to a new tube, resulting in the lightest 6.25% of cells from the original sample and this was defined as the top fraction. After rinsing three times, the top fraction ZnPP/H was measured. ΔZnPP/H was defined as the difference between the top fraction and the whole blood ZnPP/H.

The Tube Reader was custom-manufactured by UW-Madison Biomedical Engineering students as a team project. The Tube Reader was 4.7 × 2.4 × 2.0 cm (12 × 6 × 5 in), L × W × H manufactured in static dissipative ultra-high molecular weight polyethylene with placeholders drilled for 8 × 48 mm microcentrifuge tubes. The laminated scale was placed behind a Plexiglas window and adjusted to match each tubes packed cell volume (Figs S1–S3, Table S1, supplemental materials online at www.pedresearch.org).

An aliquot of blood obtained before reticulocyte enrichment was stained for manual reticulocyte percentage with the supravital new methylene blue stain (Sigma Chemical Co., St. Louis, MO). Reticulocyte percentage was also obtained on an aliquot of the top fraction (top 6.25% of cells) after reconstitution with plasma. One of two different blinded reviewers determined the reticulocyte percentage by counting 1000 cells (500 on each of 2 different smears) at 100× power.

Data analysis. Sample size was determined to be eight per group for P12 control and mild ID rats assuming 80% power, *p* value <0.05, to measure a 50 μmol/mol rise in ZnPP/H with top fractionation. For humans, nine per group were estimated, with 80% power, *p* value <0.05. Top ZnPP/H from aliquots of the same sample within-day and between-day were examined by repeated measures ANOVA. Paired, unpaired *t* testing, Kruskal-Wallis, or factorial ANOVA, with Fisher's *post hoc* testing was used. Values reported are mean ± SEM.
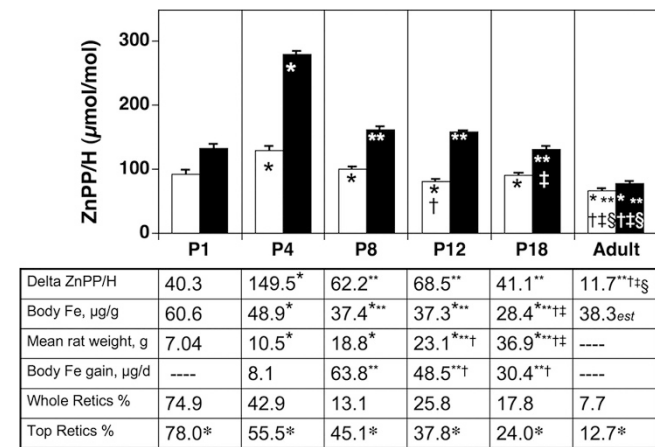
RESULTS

Reproducibility of top fraction ZnPP/H. The minimal initial sample volume necessary for top fraction ZnPP/H was 300 μL. Consistent with the literature (19,21,22), we previously showed that aliquots of whole blood ZnPP/H assayed within- or between-days for up to 10 subsequent days were similar (23). If stored at 4°C, aliquots assayed for top fraction ZnPP/H were also similar *via* repeated measures ANOVA in human and rat samples within-days or between-days for 10 d. Multiple aliquots of the same sample assayed four times on the same day for top fraction ZnPP/H were similar ($n = 9$, $F_{8,24} = 1.52$), $p = 0.23$. Multiple aliquots of the same sample assayed over three different days for top fraction ZnPP/H were similar ($n = 9$, $F_{8,16} = 0.755$), $p = 0.48$. Mean coefficient of variation on individual repeated samples was 9.6%
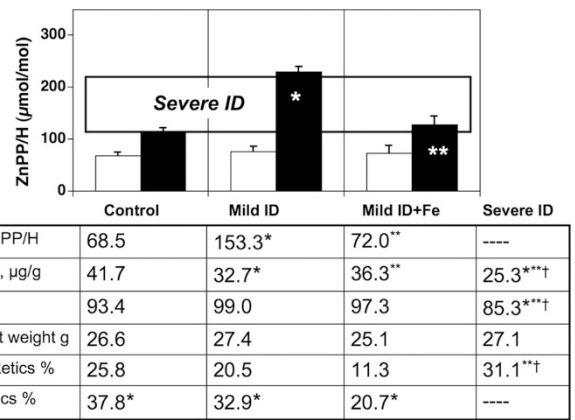
(range 1.4–26%). In a subset of P4 rats ($n = 4$), we determined whether sample processing produced an artifactual elevated ZnPP/H by examining the heaviest fraction ZnPP/H. P4 rats were selected because these rats exhibited the greatest ΔZnPP/H (greatest difference between whole blood ZnPP/H and top ZnPP/H). We observed no difference between ZnPP/H from the heaviest 75% (103.5 μmol/mol) and whole blood ZnPP/H (94.0 μmol/mol), but the lightest top 6.25% fraction was higher (228 μmol/mol), $p < 0.002$.

Developmental differences in ZnPP/H and utility of ΔZnPP/H in newborn rats. In normative data from the rat developmental study, whole blood or top fraction ZnPP/H differed by postnatal age (Fig. 2). The top fraction ZnPP/H was higher than whole blood ZnPP/H at all ages, except in adult rats, with top and whole blood being similar. ΔZnPP/H, body iron concentration (μg g^{-1}), body weight (g), daily body Fe increment (μg d^{-1}) and mean reticulocyte percentages (whole/top fraction) differed over development, shown in the table inset within Figure 2. Suckling and weanling rats exhibited higher ΔZnPP/H than adult rats. However, ΔZnPP/H in suckling and weanling rats was similar to each other, except at P4, when higher ratios accompany the lowest daily body increment and greatest fall in body iron concentration (Fig. 2).

As anticipated, mean Hb and mean body iron concentration in P12 rats with longstanding severe ID were lower than controls, mild ID, or mild ID+Fe (Fig. 3). Because whole blood ZnPP/H in P12 rats with severe ID was markedly higher than controls, the top fraction or ΔZnPP/H was not studied. Hb values and ZnPP/H in P12 rats with mild ID and mild ID+Fe were similar to controls. However, the top fraction or ΔZnPP/H allowed sufficient discrimination of the lower body iron content seen in mild ID, compared with controls or mild
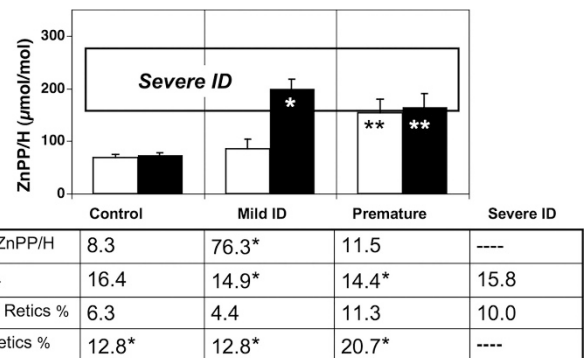

Figure 3. Whole blood (*white*) and top fraction (*black*) ZnPP/H in P12 rats control rats ($n = 14$), rats with mild ID ($n = 7$), or rats with mild ID+Fe ($n = 8$). Mean ± 2 SD whole blood ZnPP/H in severe ID ($n = 15$), shown in the horizontal white box, differed from control, $p < 0.0001$. Note that dam-fed P12 controls were handled daily and are not the same rats described in the unmanipulated developmental study above. ΔZnPP/H and body Fe concentration differed by treatment, $p < 0.005$, but rat weight was similar. Top and ΔZnPP discriminate mild ID from control and mild ID+Fe. For each individual measurement on the figure or table, *post hoc* comparisons are shown, with values differing from control indicated by *, from mild ID by **, and mild ID+Fe by †, $p < 0.05$. In the bottom two rows of the table, reticulocyte (retics) percentages in each group rose, showing an enhancement of immature erythrocytes, as indicated by the star (*), $p < 0.05$.

	Control	Mild ID	Mild ID+Fe	Severe ID
Delta ZnPP/H	68.5	153.3*	72.0**	----
Body Fe, μg/g	41.7	32.7*	36.3**	25.3***†
Hb g/L	93.4	99.0	97.3	85.3***†
Mean rat weight g	26.6	27.4	25.1	27.1
Whole Retics %	25.8	20.5	11.3	31.1**†
Top Retics %	37.8*	32.9*	20.7*	----

ID+Fe (Fig. 3). Mean reticulocyte percentages (whole/top fraction) are also shown in the table inset within Figure 3.

Developmental differences in ZnPP/H and utility of ΔZnPP/H in human cord blood. In healthy term newborns, there was no difference between whole and top fraction ZnPP/H. In human premature newborns without conditions associated with impaired iron status, whole blood and top fraction ZnPP/H was higher than control term newborns, but no difference between whole and top fraction (Fig. 4). Whole blood ZnPP/H in mild ID was similar to control, but ΔZnPP/H

	P1	P4	P8	P12	P18	Adult
Delta ZnPP/H	40.3	149.5*	62.2**	68.5**	41.1**	11.7**†‡§
Body Fe, μg/g	60.6	48.9*	37.4***	37.3***	28.4***†‡	38.3*est*
Mean rat weight, g	7.04	10.5*	18.8*	23.1***†	36.9***†‡	----
Body Fe gain, μg/d	----	8.1	63.8**	48.5**†	30.4**†	----
Whole Retics %	74.9	42.9	13.1	25.8	17.8	7.7
Top Retics %	78.0*	55.5*	45.1*	37.8*	24.0*	12.7*

Figure 2. Whole blood (*white*) and top (*black*) ZnPP/H in P1 newborn ($n = 14$), P4 suckling ($n = 13$), P8 suckling ($n = 14$), P12 suckling ($n = 15$), P18 weanling ($n = 11$), and adult rats ($n = 10$). Whole blood and top ZnPP/H differ by postnatal age as shown in the figure, $p < 0.0001$. In the inset table, ΔZnPP/H, body Fe concentration, rat weight, body iron increment also differ by postnatal age, $p < 0.0001$. Normal body iron for adult rats was estimated (est) based on Hb and that 75% of body iron is contained in Hb. For each individual measurement in the figure or table, *post hoc* comparisons are shown, with values differing from P1 indicated by *, from P4 by **, P8 by †, P12 by ‡, and P18 by §, $p < 0.05$. In the bottom two rows of the table, reticulocyte (retics) percentages in each group rose, showing an enhancement of immature erythrocytes, as indicated by the star (*), $p < 0.05$.

	Control	Mild ID	Premature	Severe ID
Delta ZnPP/H	8.3	76.3*	11.5	----
Hb g/L	16.4	14.9*	14.4*	15.8
Whole Retics %	6.3	4.4	11.3	10.0
Top Retics %	12.8*	12.8*	20.7*	----

Figure 4. Whole blood (*white*) and top fraction (*black*) ZnPP/H in cord blood of control ($n = 12$) and mild ID ($n = 8$). Mean ± 2SD whole blood ZnPP/H in severe ID ($n = 11$) is shown in the white box, differs from control, $p < 0.0001$. Although whole blood ZnPP/H is similar, ΔZnPP/H is greater in mild ID, compared with controls, $p < 0.001$. Although whole blood ZnPP/H in premature infants is greater than in controls, ΔZnPP/H is similar to controls. For each individual measurement on the figure or table, *post hoc* comparisons are shown, with values differing from control indicated by *, and from mild ID by **, $p < 0.05$. In the bottom two rows of the table, reticulocyte (retics) percentages in each group rose, showing an enhancement of immature erythrocytes, as indicated by the star (*), $p < 0.05$.

in mild ID was higher than controls (Fig. 4). Whole blood ZnPP/H from the severe ID group (IDM group) known for risk of tissue iron depletion was higher than control term newborns. Mean reticulocyte percentages (whole/top fraction) and mean Hb values are also shown in the table inset within Figure 4.

DISCUSSION

This is the first study to show that measuring ZnPP/H in the least mature erythrocytes from newborn rats or humans improves the sensitivity of ZnPP/H to detect impaired iron delivery. Although examining top fraction ZnPP/H is informative, examining ΔZnPP/H removes overlaps between normal and abnormal and lessens the developmental differences in newborn rats and humans. Whole blood ZnPP/H was previously shown to measure erythrocyte iron incorporation in umbilical cord blood and NICU neonates (3,4,8). ZnPP/H is elevated in conditions known to exhibit impaired tissue iron, including IDMs. Erythrocytes circulate for up to 90 d in newborns (24). With maturation, erythrocytes progressively increase in density (25). Reticulocytes, the most immature erythrocytes, are identifiable by supravital staining of retained cytoplasmic RNA, mitochondria, ribosomes, centriole, and Golgi bodies (26). Reticulocyte enrichment has been described for years, including Percoll-Renografin gradients, phthalate ester separation, silicone oil gradients, and employing high-speed/longer duration floor model centrifuges for density separation (11,16,20,25). With these complex methods, the least dense cells immature cells can be concentrated up to 10 times, using reticulocyte percentages as indicators. We selected a simpler, lower speed centrifugation using universally accessible microcentrifuges to accommodate small (300 μL) sample volumes. Although other low density immature erythrocytes remain in the top fraction and contribute to the top fraction ZnPP/H, we measured reticulocytes to indicate separation (16,20,25) because reticulocytes were sufficiently higher (doubled).

In the rat developmental study, normative data show that ZnPP/H changes with postnatal age, but the highest ZnPP/H and largest ΔZnPP/H after virtually no net 4-d body iron accretion. This observation supports our previous published data showing limited erythrocyte iron delivery in normal rats dam-fed at P4–P6 (17). It is unclear why normal newborn rats experience apparently insufficient iron delivery at P4. However, because growth is inversely proportional to litter size, it is possible that experimental culling of 12–14/litter to 10/litter, allows pup growth to exceed the pup's ability to mobilize sufficient iron. The relatively lower ZnPP/H and ΔZnPP/H at P8, P12, and P18 accompanied increased iron accretion. Because adult rats exhibited the lowest ΔZnPP/H, our data support that the relatively higher ΔZnPP/H in normal developing rats could be the relative faster growth rate and greater iron needs for erythropoiesis, compared with developing humans. For rats in the treatment studies, Hb and body iron levels confirm the presence of mild or severe ID. Severe ID rats exhibited relatively higher whole blood ZnPP/H, but ratios in control, mild ID or mild ID+Fe rats were similar.

The advantage of ΔZnPP/H is to discriminate a difference between untreated mild ID from control and mild ID+Fe.

In healthy newborns, we anticipated and observed no appreciable ΔZnPP/H, reflecting normal fetal erythropoiesis at term. The severe ID (IDM) group was included to demonstrate that ΔZnPP/H is unnecessary when whole blood ZnPP/H is markedly abnormal. In two-thirds of IDM, impaired transferrin receptor-mediated iron transport in placenta, fetal hypoxia, exaggerated growth, and exaggerated erythropoiesis (8,23,27) result in impaired tissue iron. In IDM, either ZnPP/H (4,8) or erythrocyte protoporphyrin (28) were correlated with disturbed iron status, and to surrogates of maternal glycemic control (23,28). From mothers at-risk for iron deficiency, we defined the mild ID group as the subset with the lowest quartile plasma ferritin levels, but normal whole blood ZnPP/H. In cord blood, using ΔZnPP/H improved the sensitivity of ZnPP/H to detect mild ID. Previous work shows that whole blood ZnPP/H is inversely related to gestation (4,8), but ΔZnPP/H does not require gestation-based normals. We examined premature infants to show that, although whole blood ZnPP/H was higher than at term, absence of appreciable ΔZnPP/H supports normal fetal erythropoiesis.

Other potential clinical indicators of iron status in newborns have been investigated. A soluble truncated transferrin receptor, used in older patients, was more strongly correlated with erythropoiesis than iron indices in newborns (29–31). Interpretation of erythrocyte indices, including mean corpuscular volume and red cell distribution width, is more difficult in the newborn, due to developmentally higher mean corpuscular volume and red cell distribution width in newborns, making these measures less informative in the newborn (32). Reticulocyte indices (reticulocyte Hb or percent hypochromic reticulocytes), available on newer clinical flow cytometer analyzers, are effective at identifying preanemic mild ID in infants (1,8,15). Although measuring different characteristics of the erythrocyte, in studies reporting both reticulocyte Hb and ZnPP/H, results are comparable when diagnosing ID *versus* normal erythropoiesis (13,14) in children. Although our method was designed for simple nonautomated hematofluorometers, a channel measuring reticulocyte ZnPP/H could be also developed for automated clinical flow cytometry analyzers, as suggested by Labbé and Dewanji (33).

Because reticulocyte indices are not universally available, whole blood ZnPP/H is simple, cost-effective, and requires minimal sample volume when screening iron status of premature infants (9,10). Ratios are directly measured by clinical hematofluorometers on drops of blood with minimal cost (34,35). Changes in ZnPP/H are relatively specific, with lead poisoning, unlikely in newborns and hospitalized premature infants, raising ratios (19). In newborns, jaundice or pigmented drugs may artifactually elevate ZnPP/H, but simple rinsing removes plasma pigments (19). We found that reticulocyte enrichment step was reproducible and ΔZnPP/H relatively more sensitive than whole blood ZnPP/H in determining preanemic mild ID in newborn rats and humans. Four additional observations supported that ΔZnPP/H was not artifact. First, the most common disturbance of red cell processing is hemolysis, but we observed that ZnPP/H falls with hemolysis

(unpublished data). Second, we mathematically projected that the bottom fraction ZnPP/H would be unchanged after centrifugation and found that it was unchanged. Third, ΔZnPP/H was unappreciable in normal term and preterm humans and adult rats. Fourth, immature erythrocytes comprise nearly all cells in the top fraction. Previous reports describe that adult whole blood also contains a very small percentage ($<0.1\%$) of low-density mature cells identified by resistance to shrinking with valinomycin (36). We treated neonatal cord blood with valinomycin to identify these mature low-density in the top fraction, but found scant numbers (data not shown).

Measuring whole blood and ΔZnPP/H may minimize the disadvantage of developmental differences in whole blood ZnPP/H in rats and humans minimize the overlap between normal and abnormal values and improve sensitivity in identifying mild ID. We speculate that identifying mild ID by ΔZnPP/H when treating the anemia of prematurity could be clinically useful. Miller *et al.* showed that whole blood ZnPP/H may respond to iron therapy (9), but by employing whole and ΔZnPP/H would potentially improve the sensitivity to observe an iron response, and when iron dose is titrated to ZnPP/H (9), avoid over treatment. Future work in this field is necessary.

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