Hormonal Regulation of Iron Partitioning During Pregnancy

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Hormonal Regulation of Iron Partitioning During Pregnancy

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Iron deficiency is the most prevalent nutrient deficiency worldwide. Pregnant women are at an increased risk of iron deficiency, and this condition has been associated with adverse maternal and neonatal outcomes. Despite the magnitude and known consequences of iron deficiency during pregnancy and at birth, much is left to learn about iron homeostasis during these life stages. It is increasingly appreciated that iron homeostasis is under hormonal control, namely erythropoietin, erythroferrone and hepcidin, however normative concentrations at birth and changes in these hormones during pregnancy and are not fully understood. The overall goal of this research was to characterize maternal iron regulatory hormones across pregnancy, within the placenta, and at birth, highlight challenges with interpretating iron status biomarkers, quantify iron transfer to the fetus from different maternal sources and explore determinants of iron partitioning between the maternal and neonatal compartment.

A main novel focus of this dissertation is on the recently identified hormone, erythroferrone. Concentrations of this hormone were characterized for the first time across gestation, in placental tissue obtained at delivery and within the neonatal compartment using umbilical cord blood collected at birth. The study utilized two pregnancy cohorts, one comprised of women carrying multiple fetuses (n = 83) and the other comprised of pregnant adolescents (n = 255). Maternal and neonatal

erythroferrone was associated with hemoglobin and erythropoietin in the respective compartments, however differences in erythroferrone's relationship with hepcidin were evident between pregnant women and their neonates. Erythroferrone was not a strong predictor of maternal or neonatal iron status or anemia, however erythropoietin and the ratio between hepcidin/erythropoietin appeared to perform superior. All placentae collected from these women and adolescents expressed all three hormones, including those delivered as early as 25 weeks of gestation. Only placental erythroferrone and erythropoietin appeared to be associated with maternal, placental or neonatal iron status, and different relationships between these placental transcripts and maternal/neonatal iron status existed between the two cohorts. In an additional cohort of 15 women, stable iron isotopes (^{57}Fe and ^{58}Fe) were used, one to label the maternal RBC pool early in pregnancy and one to label dietary iron in the third trimester to assess relative transfer of iron from these sources to the fetus. Iron absorption increased as pregnancy progressed and was strongly related to maternal iron status, as was transfer of both iron sources to the neonatal compartment. This study also demonstrated that the lifespan of the red blood cell during human pregnancy appears to be shorter than adult men and non-pregnant women. Women with increased iron demand demonstrated greater iron absorption, shorter red blood cell lifespan and transfer of more iron from red blood cell catabolism to their developing fetus. Future research should be focused on understanding the utility of erythropoietin in identifying women with increased iron need and differential partitioning of iron between pregnant adult women and adolescents.

BIOGRAPHICAL SKETCH

Katherine Delaney was born in Orchard Park, NY on February 16th, 1992 to Mary and Michael Delaney. Katherine graduated from Hamburg High School in 2010 and continued her education at Hobart and William Smith Colleges, obtaining a B.S. in biochemistry with a minor in health professions in 2014. While studying as an undergraduate, she worked with Dr. Walter Bowyer and Dr. Erin Pelkey in the Chemistry Department and Dr. Rahul Kanadia in the Physiology and Neurobiology Department at The University of Connecticut. Unsure on what field to pursue for graduate school, she spent two years post-graduation as a post-baccalaureate fellow at the National Institutes of Health (NIH) working with Dr. Hong Xu in a Molecular Genetics Laboratory. While at the NIH, she was exposed to clinical research and the role of nutrition in human health. Katherine entered the Ph.D. program in Human Nutrition in the Division of Nutritional Science at Cornell University in 2016, where she joined Dr. Kimberly O'Brien's research group. Her dissertation focuses on understanding hormonal regulation of iron homeostasis during pregnancy, in the placenta and at birth.

DEDICATION

This dissertation is dedicated to my family and friends who, through their love and encouragement, helped me achieve this great accomplishment. I would also like to dedicate this to my nieces and nephews who were born during my graduate school experience and have grown so much during this time: Eddy, Bodhi, Sloan, Ro and Finn. I hope they believe in themselves and know that they can achieve their greatest dreams.

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I am appreciative for all of the women, teens and their newborns that participated in the studies and for the funding sources that made these projects possible. I am also grateful for the past graduate students, collaborators, doctors and research coordinators who spent incredible amounts of time and effort collecting the data I had the opportunity to expand on for my dissertation.

Finally, I like to thank my family for their love and encouragement. Although this pathway was unconventional, they supported me every step of the way and believed that I could achieve this long standing goal.

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LIST OF ABBREVIATIONS

CRP	C-Reactive Protein
DMT1	Divalent Metal Transporter 1
EPO	Erythropoietin
ERFE	Erythroferrone
Fe	Iron
Fpn	Ferroportin
Hb	Hemoglobin
HIF	Hypoxia-Inducible Factor
ID	Iron Deficiency
IL-6	Interleukin 6
IRE	Iron Responsive Element
IRP	Iron Regulatory Protein
LBW	Low Birth Weight
RBC	Red Blood Cell
SF	Serum Ferritin
SGA	Small for Gestational Age
sTfR	Soluble Transferrin Receptor
sβ	Standardized β-coefficient
TBI	Total Body Iron
TfR	Transferrin Receptor
TIMS	Thermal Ionization Mass Spectrometry
ZIP 8/14	Zrt/Irt-like protein-8/14

CHAPTER 1

INTRODUCTION

Specific Aims:

Iron deficiency (ID) is the most widespread micronutrient deficiency worldwide. Pregnant women are particularly vulnerable to ID due to the high iron demands of pregnancy. In the United States, it is estimated that 7.8 million adolescents and women of childbearing age have ID and of these, 3.3 million (42%) have ID anemia (1). The prevalence of ID increases across gestation from 7% to nearly 30% by the end of pregnancy (2). The consequences of ID on the developing fetus are increasingly appreciated. To insure both the mother and her fetus have sufficient iron during pregnancy, an adequate understanding of iron homeostasis and maternal iron status is needed. Currently, indicators such as serum ferritin and soluble transferrin receptor that are used to assess iron status during pregnancy are either not standardized across assays or are influenced by inflammation, which changes across pregnancy. Due to these limitations, using serum ferritin and/or soluble transferrin receptor may underestimate the burden of ID during pregnancy (3).

Iron homeostasis is under hormonal control, and to date three iron regulatory hormones have been identified: erythropoietin (EPO), erythroferrone (ERFE) and hepcidin. While data on EPO and hepcidin during human pregnancy have been published, a commercial assay for ERFE was only released in 2017 and at this time, maternal and neonatal data on this hormone are lacking. Erythroferrone may be a strong indicator of maternal erythropoietic iron demand and this hormone is not impacted by inflammation. The developing fetus also produces its own iron regulatory hormones, but questions remain on the interpretation of these biomarkers in umbilical cord blood. Furthermore, a greater understanding of placental iron transport

mechanisms, and how these are altered during maternal or fetal ID is needed. Finally, more information is needed on how variable sources of maternal iron (recent diet, iron stores or red blood cell catabolism) are used to support fetal iron demands.

This doctoral project has applied a comprehensive approach to address existing gaps in knowledge on maternal and neonatal iron partitioning. The overall goal of this project was to better understand physiological adaptions in iron homeostasis that occur across gestation. The focus has been placed on the use of iron regulatory hormone ERFE as an iron status biomarker, sources of maternal iron that are used to supply the fetus, relative partitioning of iron between mother and fetus, placental expression of iron regulatory hormones and interpretation of iron status biomarkers in umbilical cord blood. These findings will advance the field of maternal nutritional physiology, better define iron trafficking across the placenta, and help characterize iron status indicators in the newborn.

Specific Aims and Hypothesis

Aim 1: Elucidate maternal iron homeostasis by characterizing iron regulatory hormones across gestation, characterizing the sources of iron that are transferred to the fetus and evaluate iron partitioning between the maternal, neonatal, and placental compartments.

Aim 1a. Characterize erythroferrone (ERFE) concentrations across pregnancy in relation to previously characterized iron status indicators and hormones and clarify the predictive ability of all three iron regulatory

hormones in identifying women with gestational ID and anemia. *Hypothesis: ERFE will be positively associated with EPO, although EPO will provide greater predictive ability for identifying women with ID, IDA, and anemia.*

Aim 1b. Utilize stable iron isotopes to evaluate partitioning of dietary iron between the maternal, neonatal, and placental compartments. *Hypothesis: Maternal utilization of dietary iron will be greater than fetal utilization and fetal utilization will be greatest in women with low iron status.*

Aim 1c. Evaluate longitudinal changes in maternal iron absorption across pregnancy, and how maternal and fetal iron needs are met by red blood cell iron catabolism or absorption from recent diet. *Hypothesis: Iron absorption will increase as gestation progresses. Women with lower iron stores will have higher iron absorption, shorter red blood cell lifespan and iron released from red blood cell catabolism will serve as a large iron source for the fetus.*

Aim 2: Elucidate placental iron homeostasis by measuring placental ERFE mRNA and evaluating associations with maternal and neonatal iron status and regulatory hormones.

Aim 2. Evaluate ERFE mRNA expression in placental tissue obtained from pregnant adolescents and women carrying multiples to evaluate maternal and neonatal determinants of the placental ERFE expression and to evaluate possible correlations. *Hypothesis: Placental ERFE production will be associated with maternal and neonatal hemoglobin*

concentration as well as placental EPO expression. Expression of placental ERFE transcript will differ between the two cohorts.

Aim 3: Elucidate neonatal iron homeostasis by characterizing umbilical cord iron regulatory hormones in relation to neonatal iron status.

Aim 3a. Highlight challenges with interpreting newborn hemoglobin relationships with iron status biomarkers in umbilical cord blood. *Hypothesis: Many assume that interrelationships between iron status indicators in umbilical cord blood serum mirror those observed in older children and adults. Reanalysis of data generated from neonates born to pregnant adolescents and adult women carrying multiples will demonstrate these relationships are not the same.*

Aim 3b. Characterize neonatal ERFE concentrations in relation to previously characterized iron status indicators and iron regulatory hormones in umbilical cord blood. *Hypothesis: Umbilical cord ERFE concentrations will be positively associated with cord EPO, and negatively associated with cord hepcidin. Umbilical cord ERFE concentrations will be higher than maternal ERFE, and be significantly positively associated with maternal ERFE.*

Background & Significance

I. Maternal Iron Physiology During Pregnancy

General Iron Physiology

Iron is essential for cellular processes such as hemoglobin synthesis, oxygen transport, and mitochondrial function due to its unique redox potentials (4). In excess, however, it can cause oxidative damage and exacerbate disease states (5). As there are no physiological means of excreting excess iron, to avoid iron overload the body must rigorously control net iron absorption, release of iron from stores, and iron release from red blood cell (RBC) catabolism. Dietary iron absorption takes place in duodenal enterocytes through the metal ion transport protein, divalent metal transporter 1 (DMT1). Once in the enterocyte, iron can either be sequestered in the storage protein ferritin or shuttled out of the cell into circulation by the only non-heme iron export protein ferroprotein (Fpn). Trace amounts of ferritin are released into plasma in concentrations that are thought to reflect total body iron stores (6). In non-pregnant individuals 1μg/L of serum ferritin (SF) is equivalent to ~8-10 mg body iron (7, 8). Once exported through Fpn, two iron molecules bind to apo-transferrin creating a diferric-transferrin complex that circulates safely throughout the body. Tissues requiring iron express transferrin receptor (TfR) to bring the diferric-transferrin complex into the cell via receptor mediated endocytosis (9). Iron homeostasis can be regulated at the cellular level via iron regulatory proteins (IRP) and iron responsive elements (IRE). Iron responsive elements are found at either the 3' or 5' end of iron regulated genes. Genes with IRE's at the 3'end (such as TfR) are stabilized when IRP iron regulatory proteins bind, thus increasing the expression of these genes. Genes

with IRE's at the 5' end (such as ferritin), IRE-IRP interactions block translation of the mRNA leading to a decrease in the expression of these genes.

Iron homeostasis is also regulated at the systemic level by three hormones. The first hormone, erythropoietin (EPO), was identified in the 1950's (10) although its role as an erythroid regulator of iron homeostasis was not elucidated until the 1960's (11). Erythropoietin is produced by the kidneys in response to hypoxia and stimulates the bone marrow to increase the mobilization of iron for erythropoiesis (12). This pathway is mediated by hypoxia-inducible transcription factors (HIF), that are regulated by oxygen concentrations and iron dependent oxygluterate (12). To increase circulating iron for erythropoiesis, erythropoietin stimulates the bone marrow to produce erythroferrone (ERFE) which works to rapidly suppress expression of the hepatic hormone hepcidin (15-17). Hepcidin binds to its receptor, Fpn, leading to its internalization and degradation, limiting the release of iron from various cell types (14), and its expression is increased in response to elevated iron stores or in the presence of inflammation (13, 14).

Iron Assessment in Pregnancy

Complex physiologic adaptions occur during pregnancy to ensure sufficient iron for the mother and her developing fetus while concurrently preventing iron overload (18). Iron demands increase across gestation to support maternal RBC production and growth of the placental/fetal unit. Iron requirements during pregnancy increase from 0.8 mg/day in the first trimester to over 7 mg/day in the third trimester; compared to the non-pregnant requirements of 1-1.5mg/day (19). As pregnancy progresses, both maternal and fetal erythropoiesis rates (20) and TfR concentrations

increase (21-23). Serum ferritin concentrations decrease as pregnancy progresses due to hemodilution and transfer of iron to support the demands of the placenta and fetus (23). The only published national data on changes in iron regulatory indicators in pregnancy were obtained from the cross-sectional National Health and Nutrition Examination Surveys. However, this database only evaluated hemoglobin (Hb), TfR and SF in ~500 pregnant woman (24).

The consequences of ID during pregnancy are increasingly appreciated. Anemia and ID have been associated with premature birth (25), increased prevalence of low birth weight (26) and neurodevelopmental delays (27). Accurate assessment and detection of ID during pregnancy is necessary to reduce these adverse outcomes. A basic description of iron status indicators and their cutoff levels in pregnancy and advantages/disadvantages are presented in **Table 1.1**. Hb is used to diagnose anemia and Hb cut-offs differ by smoking status, elevation, age, race, and stage of gestation (28, 29). Deficits in Hb production can be caused by a number of nutrient deficiencies including iron vitamin D, zinc, selenium, copper, vitamin A, folate, and vitamin B_{12} (30-32). An accurate diagnosis of ID requires evaluation of iron status biomarkers. Bone marrow staining of iron is the gold standard method to detect ID as ID is associated with reduced iron stores in bone marrow. However, this measure is invasive and thus isn't used as a screening method. Iron status can also be monitored using serum ferritin (SF) concentrations. Strong associations have been found between bone marrow iron and SF, and, SF is clinically utilized as a biomarker of iron stores (7). Serum ferritin measurements, however, are confounded by inflammation, making its utilization in accurately assessing ID without concurrent measures of inflammation

difficult. Further information on iron status indicator utilization can be found in these reviews (9, 33).

Table 1.1: Iron status indicators: function, clinical cutoffs during pregnancy, advantages, and disadvantages

Iron indicator	Function of indicator	Cut-offs in pregnancy for anemia and ID	Advantages	Disadvantages
Hemoglobin	Measure of anemia and proxy of functional iron	<110 g/L 1st and 3rd trimester <105 g/L 2nd trimester	Inexpensive and universally available	Low sensitivity and specificity
Hematocrit	Ratio of red cells to blood volume	<33% 1st and 3rd trimester <32% 2nd trimester	Inexpensive and quickly measured	Low sensitivity and specificity
Bone Marrow Iron	Storage iron	-	Gold standard	Highly invasive and expensive
Soluble Transferrin Receptor	Tissue iron availability	>8.5 mg/L	Quantitative and unaffected by inflammation	Lacks assay standardization
Serum Ferritin	Storage iron	<12 μg/L	Quantitative and standardized	Influenced by inflammation
Serum Iron	Circulating transferrin-bound iron	-	-	Affected by recent dietary iron, circadian rhythms, inflammation, and infection
Total Body Iron (log (TfR /SF) – 2.8229]/0.1207)	Indicator of storage and available iron	0 mg/kg	Validated in adults	Not validated in pregnant women

Iron Regulatory Hormone Changes Across Pregnancy

During pregnancy physiological adaptions occur with EPO and hepcidin in support of maternal plasma volume expansion and to provide iron for the development of the fetal/placental unit. As pregnancy progresses, EPO concentrations increase to

support expanding maternal erythropoiesis (23, 34-40). Hepcidin concentrations decrease (41) and animal data suggest hepcidin is downregulated early in gestation (42, 43) but the mechanism by which this occurs has not been elucidated. Due to previous lack of a validated human assay to measure ERFE (44), normative data on this hormone over pregnancy are lacking and our knowledge of the gestational changes in this hormone are available only from animal models. In animal models, ERFE deficient mice do not have a distinct phenotype, but are unable to suppress hepcidin after blood loss, suggesting a role during stress erythropoiesis (15). Furthermore, ERFE is essential for recovery from anemia of inflammation and is responsible for iron overload in a mouse model of the hemoglobinopathy β-thalassemia (16, 45). Erythroferrone would be expected to be greatly elevated in pregnant women with anemia, proportionate to the increases in EPO. In turn, ERFE likely plays a role in increasing iron supply for maternal erythropoietic adaptations and fetal development during pregnancy.

The ability of hepcidin to identify pregnant women with ID has been assessed (46-50), however this hormone also functions as an acute phase protein, limiting its utility when inflammation is present. Few studies have analyzed maternal EPO in relation to maternal iron status, however previous data from the O'Brien lab have found that maternal EPO is a strong determinant of iron status in two large healthy pregnant populations (51, 52). Due to the positive relationship between EPO and ERFE, ERFE may also serve as a strong iron status indicator across pregnancy, and may be superior to current indicators as it is not affected by inflammation (15). Further studies are needed to assess ERFE concentrations across pregnancy and assess the

utility of these hormones and ratios between these hormones as indicators of ID during pregnancy.

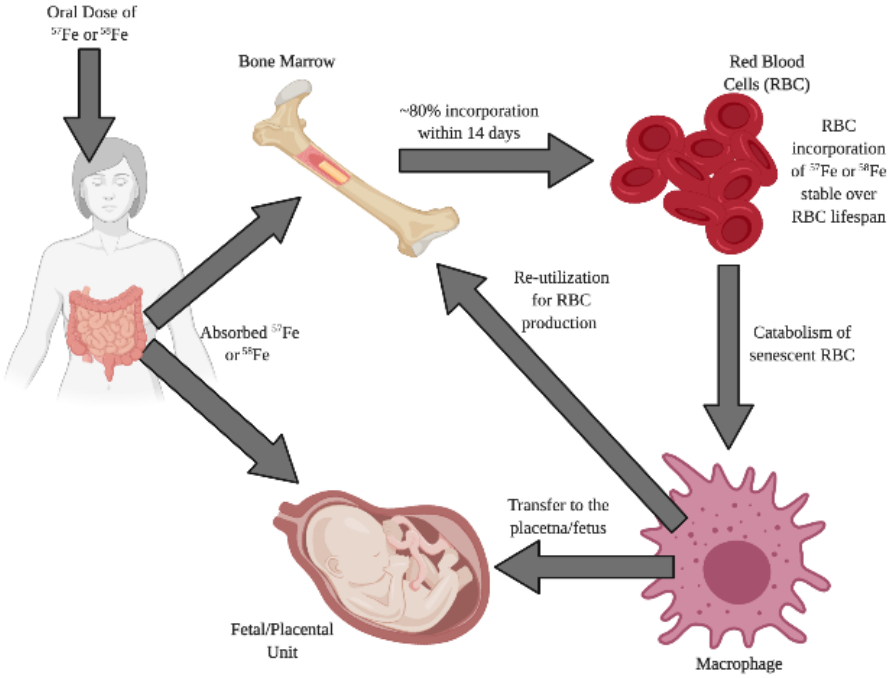
Methods to Assess Iron Utilization

Stable iron isotopes have proved to be a valuable tool for examining iron absorption and transfer throughout the body and they are a safe alternative to radioactive iron tracers used in the past. Four stable iron isotopes (^{54}Fe, ^{56}Fe, ^{57}Fe and ^{58}Fe) are naturally present in the environment and three of these are found at low fractional abundances in our bodies (^{58}Fe -0.29%, ^{57}Fe-2.14% and ^{54}Fe-5.80%). During pregnancy, stable iron isotopes can be given orally and/or intravenously to women to assess iron absorption, erythrocyte iron incorporation, and placental iron transfer and retention. These stable iron isotopes can be quantified using magnetic sector thermal ionization mass spectrometry (TIMS). **Figure 1.1** demonstrates a schematic of iron absorption, incorporation into RBC, and transfer to the fetal/placental unit.

Stable iron isotopes are commonly administered either orally or orally and intravenously (IV). Two different isotopes can be given orally to compare bioavailability between two different meals or supplements, or to evaluate one test food and compare this value to that obtained using a reference dose that is given in a form that is known to be well absorbed (typically as ferrous sulfate). Moreover, paired oral doses allows for each individual to serve as their own control. Intravenous stable iron isotope administration allows for the direct assessment of RBC iron incorporation, as it bypasses the gut and is in essence 100% absorbed. Oral doses of stable isotopes are useful for determining changes in iron absorption across pregnancy

if one assumes that RBC iron incorporation remains constant across gestation. Normative data on iron absorption at each trimester can help determine the quantity of dietary iron intake needed to meet iron demands at each gestational stage.

Figure 1.1: Iron Isotope Absorption and Erythrocyte Incorporation Modified from IAEA (53).



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stable isotopes are useful for determining changes in iron absorption across pregnancy if one assumes that RBC iron incorporation remains constant across gestation. Normative data on iron absorption at each trimester can help determine the quantity of dietary iron intake needed to meet iron demands at each gestational stage.

Stable iron isotope studies have radically improved our understanding of iron homeostasis during pregnancy. The O'Brien lab has previously utilized stable iron isotopes to assess bioavailability of both heme and non-heme iron during pregnancy, and found that heme iron is preferentially absorbed and utilized in comparison to non-heme (54, 55). Moreover, this method has helped determine that non-heme iron absorption in the 3rd trimester increases nearly three-fold compared to the non-pregnant state (54). Transfer of iron to the fetus was assessed in relation to iron status indicators, and dietary iron transfer to the baby is positively associated with neonatal Hb and EPO status and inversely associated with maternal SF and neonatal SF (56).

Although the use of stable iron isotopes has increased our knowledge of iron absorption during the 3rd trimester of pregnancy and iron transfer to the fetus, there are still many unanswered questions as to factors associated with the increase in iron absorption as well as which iron pool is utilized to meet fetal iron demands and how iron is partitioned between the mother, placenta, and fetus. For example, it is known that dietary iron contributes 3-4 mg of Fe per day into the maternal plasma pool, but within the 3rd trimester of pregnancy requirements increase to > 7 mg/day and dietary iron alone is not sufficient to fill these requirements (19, 57). Therefore, iron must be utilized from other sources (or body storage compartments); such as mobilization of iron from stores or remobilization of iron recycled from catabolized RBC's. Relative

use of each of these sources for iron transfer to the fetus has not been characterized, nor have factors associated with iron partitioning between the mother, her fetus, and the placenta.

Current Knowledge Gaps

Iron deficiency during pregnancy is detrimental to the developing fetus and accurate understanding of physiological changes and diagnosis of iron status during pregnancy is crucial. Although SF appears to be a good indicator of storage iron, it is elevated concurrently with inflammation, thereby misclassifying cases as non-iron deficient when inflammation is present (3, 58). A similar problem exists with hepcidin, which is also an acute phase protein. Pregnancy has been associated with increased inflammatory markers such as C-reactive protein (CRP) and interleukin 6 (IL-6) (59-61), and identification of an indicator of iron status that is not confounded by inflammation is needed. Erythroferrone expression is increased when iron is needed for erythropoiesis and this hormone is not impacted by inflammation, making it a strong candidate as an indicator of ID during pregnancy. However, normative data on ERFE during pregnancy as well as assessments of ERFE, EPO, hepcidin and ratios between these hormones in the identification of ID during pregnancy have yet to be published. In the current dissertation project, Aim 1a addresses the role of maternal ERFE concentrations and its relation to maternal iron status indicators and hormones during pregnancy, and the utility of all three hormones in identifying women at an increased risk for ID.

Adequate iron transfer to the fetus is reliant on mobilization of the maternal iron pool. As previously mentioned, maternal iron can either come from hepatic stores,

dietary sources, or iron released from catabolized RBC's. Iron absorption increases iron stores are depleted. Relative fetal use of iron from maternal stores, recent diet or RBC catabolism is currently unknown. In the non-pregnant state, RBCs have a finite lifespan of ~120 days (62), but the lifespan of RBC's during pregnancy is unknown. Recycled iron from RBC's contribute approximately 10 times more iron into the circulation (~20 mg/d) than the 1-2 mg per day typically absorbed from the diet. It has been hypothesized that recycled iron from RBCs may be a major source of iron for the fetus; however, this hypothesis has not been evaluated in humans. Therefore, in the current dissertation study, Aim 1b has been dedicated to elucidating iron partitioning between mother, fetus, and placenta and factors associated with partitioning between these compartments. Additionally, Aim 1c has been dedicated to using stable iron isotopes to measure change in iron absorption and change in RBC lifespan across pregnancy. This aim also describes the contributions of different maternal iron pools for fetal iron requirements.

II. Placental Iron Physiology and Regulation

Mechanisms of Placental Iron Uptake

Placental iron uptake is tightly regulated to ensure sufficient iron for placental function and iron transfer to the fetus, while preventing fetal ID or fetal iron overload. Circulating maternal iron is present as transferrin bound iron, iron within ferritin, or RBC iron (within the heme moiety). It is thought that transferrin bound iron is the predominant source of iron for the placenta, as removing placental/embryonic TfR results in embryonic lethality (63). Circulating maternal diferric-transferrin comes into direct contact with TfR on the apical membrane of the placental syncytiotrophoblast,

(64) which is then taken up by the placenta via receptor mediated endocytosis (65). The endocytosed vesicle is acidified releasing iron from transferrin, and the ferric iron released into the endosome is reduced to ferrous iron, potentially by the STEAP ferrireductase (STEAP3 or STEAP4), and then is exported from the vesicle by DMT1 into the cytosol (66, 67). DMT1 is localized to the placental syncytium (68) and is known to be internalized with diferric transferrin into the endosome in other tissues. However DMT1 appears to be redundant as DMTI null mice are born with normal body iron (69). Both Zrt/Irt-like protein's (ZIP) ZIP8 (70) and ZIP14 (71) have been found to be expressed within human placental tissue and may help mediate iron uptake.

Ferritin may also be taken up as a source of iron by the placenta, as the ferritin scavenger receptor class A member 5 (SCARA5) is expressed in the placenta (72). Heme iron may also be taken up by the placenta as many heme uptake and utilization proteins are expressed within the placenta: the heme/hemopexin receptor (CD91) (73), hemoglobin /haptoglobin receptor (CD163) (74), heme exporter feline leukemia virus subgroup C receptor 1 and 2 (FLVCR1 & FLVCR2)(75), Breast Cancer Resistance Protein (BCRP)(76) and heme carrier protein (PCFT/HCP1) (77). This is further supported by stable iron isotope data that found the placental preferentially transferred animal sourced heme to the fetus compared to an oral dose of non-heme iron as ferrous sulfate (56). Mechanisms of placental iron trafficking, have yet to be fully elucidated.

Mechanisms of Placental Iron Export

The placenta actively transports iron in what appears to be a unidirectional manner from the mother to the fetus (78-81). The fetus accumulates the majority (approximately 60%) of its total body iron (75 mg/kg) during the third trimester of pregnancy (82). As within other cells in the body, Fpn is the major iron export protein in the placenta. Animal studies have shown that placental Fpn is essential for the transport of iron from the mother to the fetus and reduced placental Fpn expression leads to newborn ID (83, 84). Before ferric iron can be transported through Fpn, it must be reduced to its ferrous form. Once exported through Fpn, ferrous iron must be oxidized before binding to transferrin. Ceruloplasmin is a ferroxidase that has been found to be endogenously produced by human placenta and plays a role in oxidation leading to the release of iron from the cells (85, 86). Knockout models have demonstrated that ceruloplasmin is not essential for placental iron transport (87), possibly due to the presence of the two other ferroxidases, hepheastin and Zyklopen. Hephaestin (heph) has been identified within BeWo placental culture cells (88), rat placentas (89) and human placentas (90). Knock out experiments found that heph fetal mice without heph receive less iron from their mothers than the controls (91). The final ferroxidase, Zyklopen, shares about 50% sequence homology to heph and is highly expressed in the placenta (92). Placental specific knockout studies of Zyklopen have not been performed, however global knockout of Zyklopen produce viable mice (93).The O'Brien lab has measured Zyklopen RNA and protein concentrations within human placenta and found that Zyklopen was positively associated with placental DMT1, TfR and Fpn, as well as placental heme transporters (94). Although these three

ferroxidases are found within human placenta, their exact subcellular localization and relationships with other iron trafficking proteins have not been explored. Moreover, animal models with triple knockout of these three ferroxidases are still viable (95), suggesting additional unidentified ferroxidases may exist in the placenta.

Regulation of Placental Iron Transport

Placental iron transfer to the fetus is dependent on maternal iron supply, but the placenta also appears to be responsive to fetal signals. On a cellular level, iron transport appears to be regulated by the IRE/IRP system, increasing TfR mRNA concentration during ID (96). Placental TfR appears to be responsive to iron demands of both the mother and the fetus, as both neonatal and maternal iron status have been found to be inversely associated with placental TfR (96-102). Further confirming the placenta increases iron transport during iron deficiency, stable isotope studies have found iron deficient mothers transfer more maternally ingested dietary iron to the fetus than iron replete mothers (55, 57). Cord TfR and maternal EPO are also positively associated with placental TfR concentrations, suggesting its regulation may also be in relation to neonatal erythropoietic drive (94).

Conversely, placental Fpn does not appear to be associated with IRP activity (99), and as Fpn located on the basolateral side of the syncytiotrophoblast is only accessible to fetal circulation. Animal studies have found that overexpression of fetal hepcidin leads to fetal iron deficiency and decreased placental Fpn (103-106). However, the O'Brien lab has found that human neonatal hepcidin does not appear to be correlated to placental Fpn protein concentration (94). Furthermore, factors influencing the regulation of placental Fpn have not been fully described.

Placental Hormone Expression

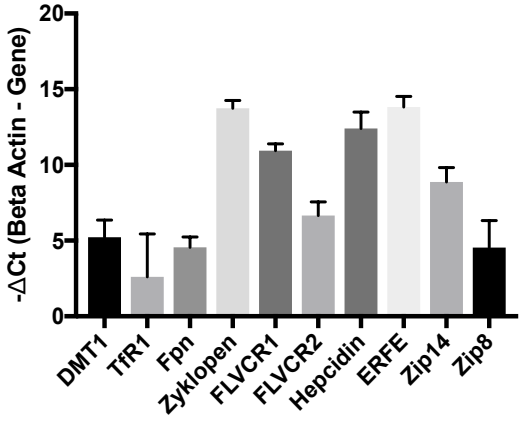
Previous studies have reported that placental EPO mRNA and protein are detected in placental tissue from animals and humans (107-109), however, these studies did not assess associations between other placental hormones or maternal and neonatal iron status. Only one study measured placenta EPO mRNA content and assessed relationships with neonatal hypoxia and EPO. The study was done in a small number of twins (n = 26) and found that placental EPO was increased in twins with abnormal uterine artery doppler and that hypofusion of severe growth restriction induces placental EPO expression (109). Erythropoietin in the fetus has been found to have non-erythropoietic roles (110), perhaps as does placental EPO.

The placenta also expresses hepcidin, and human placental hepcidin expression was not associated with placental iron transporters or maternal and neonatal iron status (94) (similar to findings within rats)(106). However, a recent study in swine did find that both placental hepcidin and Fpn were positively associated with placental iron status (111). The lack of association between placental hepcidin levels and maternal or neonatal iron status suggests that placental hepcidin may function to control placental iron concentration as well as acting as an antimicrobial peptide in the placenta, which would explain its association with placental malaria (112).

As mentioned, ERFE mediates hepcidin suppression to increase circulating iron concentrations in response to EPO stimulation. Although there appears to be no association between placental hepcidin and maternal or neonatal iron status, it is possible placental hepcidin is involved in placental iron homeostasis and thus iron

transfer to the fetus. It may also serve primarily as antimicrobial peptide in this organ. It was hypothesized that ERFE may play a role in regulating placental hepcidin, and since it is not impacted by inflammation, it may be a stronger indicator of iron transfer through the placenta. Preliminary data from 14 human placentas demonstrates that the placenta expresses ERFE (**Figure 1.2**), and that placental ERFE expression was comparable to placental hepcidin and zyklopen.

Figure 1.2: Placental expression of ERFE and iron transporter genes



Current Knowledge Gaps

The placenta plays a major role in ensuring that the fetus obtains adequate iron during pregnancy. Although many iron trafficking proteins have been found within the placenta, the localization of these proteins and their regulation is not fully understood. Moreover, whether the placenta can utilize ferritin or heme iron is unknown. Many questions remain on intracellular iron trafficking through the syncytiotrophoblast as well as the role of placental hepcidin. Erythroferrone is expressed within the placenta, but its role and relationship with placental hormones, placental iron content and gestational age is unknown. Understanding these relationships will provide additional

insight into their role and regulation within the placenta as well. As iron transport across the placenta is subject to both maternal and fetal signals, understanding placental ERFE concentrations in relation to maternal and fetal signals will be useful in understanding placental iron homeostasis. Therefore, Aim 2 has been dedicated to characterizing ERFE within the placenta and assess its relationship with maternal and neonatal iron status indicators and regulatory hormones.

III. Newborn Iron Physiology at Birth

Neonatal Iron Status at Birth

A full-term singleton pregnancy requires ~1100 mg of iron to support increased maternal erythropoiesis, as well as placental and fetal iron demands (29). Although iron is actively transported across the placenta, there are numerous conditions in which the newborn is not born with sufficient iron stores. Some of these conditions are severe maternal ID, maternal gestational diabetes, maternal hypertension, and intrauterine growth restriction (113). A recent study recruited neonates from diabetic mothers, small for gestational age neonates, and low birth weight neonates with low SF, serum iron, and transferrin saturation found infants had lower iron status at two weeks of age compared to reference data (114). Not only are neonates from complicated pregnancies at risk, recent data suggest that newborns may not have sufficient iron concentrations at birth to maintain adequate iron status during the first six months of life (115, 116). Therefore, better understanding of fetal iron accretion and neonatal iron status at birth is necessary to ensure adequate iron for development.

Neonatal Iron Status Assessment Using Umbilical Cord Blood

National data on prevalence of disease and normative data on nutritional status indicators are collected and reported in the NHANES. However, there are no normative data on iron status indicators at birth or the prevalence of anemia at birth as the NHANES program does not assess children under 1 year of age. Moreover, the American Academy of Pediatrics (AAP) does not recommend screening infants for anemia until 1 year of age unless classified as 'at-risk'. The AAP defines the 'at risk' population as those born prematurely, low birth weight, or born to women of low socio-economic status (117). The cutoffs for diagnosis of anemia in children under 1 year of age are also not agreed upon. Currently, the Institute of Medicine (IOM) recommends lowering the Hb cutoff for anemia in African American women by 0.8 g/dL and for African-American children under 5 years of age by 0.4 g/dL, although adjustments for African American newborns are not recommended at birth. Furthermore, the Centers for Disease Control (CDC) does not recommend race-specific cutoffs for children under 5 years of age (118, 119). As newborn iron absorption and metabolism mechanisms are not fully developed, interpretation of indicators shortly after birth is difficult (120).

Umbilical cord blood can be used to evaluate nutritional status at birth, without the need to sample from the newborns themselves. Many studies assess iron status at birth using umbilical cord blood and using the same cut-offs for iron status indicators as used in adults (**Table 1.2**). Neonatal anemia is assessed by umbilical cord Hb concentrations, but the cutoff concentration is higher than in adults as cord Hb concentrations are high at birth due to development in a hypoxic environment.

Neonatal iron stores at birth are commonly assessed based on umbilical cord SF, and relationships between umbilical cord SF and other iron status indicators have been assumed to be the same as observed in adults (120). Further research is needed to understand the relationships between these indicators in cord blood and how they relate to long term outcomes, as dynamic changes occur after birth. Furthermore, this would provide an opportunity to identify neonates that are an increased need for iron and intervene and prevent adverse developmental outcomes associated with early life ID.

Table 1.2: Iron Status Indicators and Cut-offs used in Umbilical Cord Blood

Iron Status Indicator	Function of indicator	Cut-off in cord blood for anemia and iron deficiency
Hemoglobin	Measure of anemia and functional iron within the body	<13.0 g/dL
Soluble Transferrin Receptor	Tissue iron availability	>8.5 mg/L
Serum Ferritin	Storage iron	<76 μg/L
Serum Iron	Circulating transferrin-bound iron	-

Iron Regulatory Hormones at Birth

Fetal erythropoiesis begins as early as 3 weeks of gestation in the yolk sak and the fetus produces its own regulatory hormones to ensure adequate iron is available to support this process. Erythropoietin is produced by the human fetus as early as 16 weeks of gestation (121), beginning in the yolk sack transitioning to the liver then the kidneys by late gestation (122, 123). Fetal production of EPO is thought to remain relatively stable during normoxic conditions (124), it is upregulated in response to

neonatal hypoxia (37, 125-128), and it is regulated by retinoic acid and thyroid hormone in an oxygen dependent manner (129, 130). It is also thought that EPO may have non-erythropoietic roles in the fetus as it has been linked to tissue protection (109, 126, 131) and immune regulation (132). Hepcidin is produced by the human fetus as early as the first trimester of pregnancy (133), it is positively associated with inflammatory markers, and elevated during infection (120, 134-137). Furthermore, the O'Brien lab and others have previously found that in healthy newborns umbilical cord blood hepcidin concentrations at birth are strongly associated with neonatal iron status (120, 134, 136-142). Since proposing this research, only one study has published ERFE concentrations in umbilical cord blood in a small cohort (n = 36) of healthy term newborns (143). However, umbilical cord EPO has been shown to be inversely associated with umbilical cord hepcidin (51, 120, 139), a relationship which may be mediated by ERFE.

Current Knowledge Gaps

Umbilical cord blood is useful for obtaining information on neonatal nutrient accretion in utero, without having to draw blood from the newborn. Numerous studies have utilized umbilical cord blood nutrient measurements to relate nutritional status at birth with longer term outcomes. Currently there are many questions regarding interpretation of iron status biomarkers in the newborn. Characterizing ERFE within umbilical cord blood and understanding the full compendium of iron regulation will help elucidate relationships between iron status indicators and hemoglobin at birth. In addition to the previously mentioned goals, Aim 3 is dedicated to characterizing umbilical cord ERFE in relation to both maternal and newborn iron status indicators.

Collectively, Aims 1 - 3 will provide a complete characterization of ERFE, including maternal expression throughout pregnancy, placental expression, and expression in umbilical cord blood and its relationship with neonatal iron status indicators and regulatory hormones at birth.

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CHAPTER 2

SERUM ERYTHROFERRONE DURING PREGNANCY IS RELATED TO ERYTHROPOIETIN BUT DOES NOT PREDICT THE RISK OF ANEMIA*

*Delaney, KM; Guillet, R; Pressman, E.K; Ganz, T; Nemeth, E; O'Brien, K.O. Serum erythroferrone during pregnancy is related to erythropoietin but does not predict the risk of anemia. Submitted to J Nutr 2021; *In Press*

Abstract

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

Objective: 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: Erythroferrone was measured in extant serum samples collected from two longitudinal cohorts comprised of adult women carrying multiple fetuses (n = 79) and pregnant adolescents (n = 218) at mid-gestation (~26 weeks) and delivery (~39 weeks). Receiver operating characteristic curves were generated to characterize the predictive ability of serum ERFE, hepcidin, 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 weeks of gestation and at delivery. ERFE was positively associated with EPO at mid-gestation ($\beta = 0.14$, $p = 0.002$, $n = 202$) 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 mid-gestation 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 ratio between hepcidin/EPO was best able to identify women with iron deficiency anemia (AUC: 0.85 and 0.84).

Conclusion: Maternal ERFE was significantly associated with EPO, but was not able to identify women with gestational iron deficiency. At term the hepcidin/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.

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, three 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 interrelationships between these hormones in human pregnancies.

In the United States, 16% of pregnant women are estimated to develop ID (6). Prevalence of ID increases roughly 4-fold from early to late gestation (7). Iron deficiency is typically identified based on measures of depleted serum ferritin (SF) or increased soluble transferrin receptor (sTfR) concentrations. Serum ferritin 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. Soluble transferrin receptor has also been used to classify ID in pregnant women (16, 17) 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. Erythropoietin 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 to values observed in non-pregnant 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 non-heme 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. Erythroferrone 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 paper was to characterize ERFE concentrations across gestation in women at increased risk of ID and IDA, and to evaluate the relationship between ERFE and two other iron regulatory hormones (erythropoietin and hepcidin). All three 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 more than one 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:

Erythroferrone was measured in extant serum collected from two pregnancy cohorts. The first cohort consisted of pregnant adolescents recruited from the Rochester Adolescent Maternity Program (RAMP) in Rochester, NY between 2006 – 2012. The second cohort included women carrying multiple fetuses that were recruited from Strong Memorial Hospital and Highland Hospital in Rochester, NY from 2011 to 2014 (**Figure 2.1**). 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.

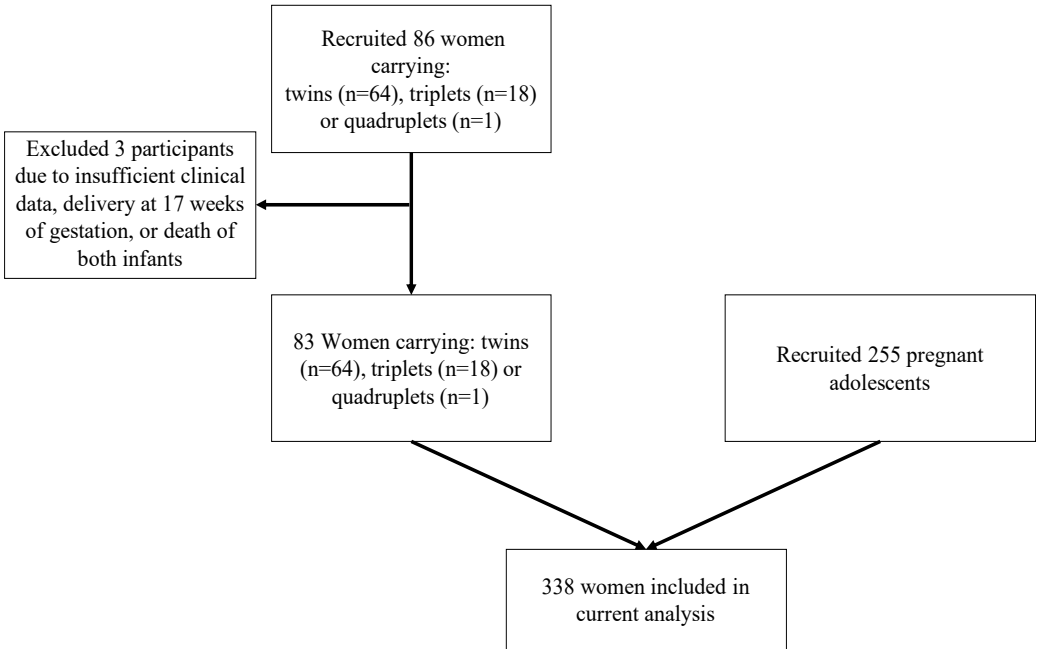


Figure 2.1: Flow Chart Mothers Included in Erythroferrone Analysis.

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. Pre-pregnancy BMI (ppBMI) was classified using CDC guidelines as either underweight (ppBMI < 18.5 kg/m2); normal weight (ppBMI ≥ 18.5 and < 25 kg/m2); overweight (ppBMI ≥ 25 and < 30); or obese (ppBMI ≥ 30 kg/m2) (44). Additionally, gestational weight gain categories were classified using IOM 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 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 (44). Gestational age (GA) 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 more than ten days, 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 women (1%) of teens in the pregnant adolescent cohort.

Serum collection and biochemical analysis:

Nonfasted maternal blood (15 mL) was collected from women at mid-gestation (~26 weeks, n = 226) and when admitted to the hospital for delivery (~39 weeks, n = 333). As this was a convenience sample, the number of blood samples collected and the timing of collection across gestation varied (**Figure 2.2**). 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, Santa Clara, CA). Anemia across pregnancy was defined for Caucasians as hemoglobin concentration < 11.0 g/dL in the first and third trimesters and < 10.5 g/dL in the second trimester and for African Americans < 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, and serum was separated and stored at -80degrees C until analysis.

Trimester 1	Trimester 2	Trimester 3	Delivery	Adolescent	Multiples
	X	X	X	N = 0	N = 13
		X	X	N = 47	N = 17
X	X		X	N = 0	N = 5
	X		X	N = 108	N = 36
X		X	X	N = 0	N = 1
			X	N = 100	N = 7
X			X	N = 0	N = 3

Figure 2.2: Timepoints of Available Samples for ERFE measurement.

Serum ERFE was measured using a validated ELISA (Intrinsic Lifesciences, La Jolla, CA). 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 below 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 inter-assay coefficient of variation, 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 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, Erlangen, Germany) with an inter-assay CV of 7.5%. Serum ferritin and serum sTfR were measured by ELISA as previously described (36, 43). Iron deficiency 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, Waltham, MA). Serum folate and serum vitamin B-12 were measured by an Immulite 2000 immunoassay system (Siemens Healthcare, Erlangen, Germany). 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 interleukin-6 (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 (La Jolla, CA) and the LOD was 5 ng/mL, and the inter-assay 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 (Torrance, CA) that had a 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 inter-assay 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, Temecula CA) and CRP was measured by immunoassay (Siemens Immulite 2000, Erlangen, Germany) (36). For the multiples cohort, IL-6 and CRP were measured by ELISA from R&D Systems (Minneapolis, MN) (43).

Statistical analysis:

Maternal characteristics are presented as mean ± SD or percent for continuous and categorical outcomes respectively. The Shapiro-Wilk test was used to assess normality of data, and non-normal 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 the Wilcoxon's rank-sum test was used to test statistical differences between non-parametric 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 relationships between iron status indicators. The relationship significance remained the same after controlling for gestational age at each time point and study population, so only unadjusted data are presented. Bartletts 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 mid-gestation 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 (%) vs 100% - specificity (%) for varying indicator concentrations. The area under the curve (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 two cohorts are presented in **Table 2.1**. Women in the multiples cohort were significantly older, entered pregnancy with a higher parity, had a higher pre-pregnancy body mass index (ppBMI), higher gestational weight gain (GWG) and a significantly shorter gestational period compared to the adolescent cohort. Overall, 15% of women were anemic at mid-gestation and 24% were anemic at delivery. Of women who were anemic, 74% had iron deficiency anemia at mid-gestation and 53% had iron deficiency anemia at delivery. At mid-gestation 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) at both mid-gestation and delivery. None of the participants studied were vitamin B-12 insufficient at mid-gestation, but at delivery 11% of those screened were found to be vitamin B-12 insufficient. Iron status of the women at mid-gestation and delivery are presented in **Table 2.2**.

Table 2.1: Characteristics of Women Carrying Multiples and Pregnant Adolescents

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/m²	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 a week (%)	21	11*	24
< 2 times a week (%)	25	7*	30
Types of multiples			
Twins (%)	77.1	77.1	-
Triplets (%)	21.7	21.7	-
Quadruplets (%)	1.2	1.2	-

Values are presented as mean ± SD or percentage. *Denotes a significant difference between cohorts at $p < 0.05$.

Table 2.2: Iron Status Indicators During Pregnancy

	Multiples Cohort		Adolescent Cohort	
Variable	**Mid-gestation**	**Delivery**	**Mid-gestation**	**Delivery**
GA (wk)	24.8 ± 5.2 (75)	34.8 ± 2.7 (83)	26.0 ± 3.4 (155)	39.3 ± 2.6 (250)
Hb (g/dL)	11.1 ± 1.4 (74)	11.2 ± 1.6 (83)	11.2 ± 0.9[a] (120)	11.5 ± 1.4 (230)
Anemia (%)	26[*] (19)	37[*] (31)	8 (10)	19 (44)
Hct (%)	33.6 ± 3.5 (75)	33.8 ± 4.0 (83)	33.3 ± 2.5 (125)	34.1 ± 3.7 (209)
sTfR (mg/L)	4.2 [3.7 – 4.8][a] (73)	4.9 [4.3 – 5.7] (61)	4.4 [4.1 – 4.7][a] (147)	4.8 [4.6 – 5.2] (208)
sTfR > 8.5 (%)	12[a] (9)	23(14)	7[a] (10)	14 (29)
SF (µg/L)	15.3 [13.0 – 18.0][a] (73)	22.2 [17.9 – 27.5] (61)	17.3 [15.0 – 19.8][a] (147)	21.5 [19.2 – 24.1] (206)
SF < 12 (%)	37[a] (27)	26 (16)	27 (39)	21 (44)
TBI (mg/kg)	3.2 ± 3.4 (73)	3.9 ± 4.4(61)	3.5 ± 3.9 (147)	3.9 ± 4.0 (206)
TBI < 0 (%)	15 (11)	18 (11)	15 (22)	15 (30)
Vitamin B-12 (pmol/L)	390 [320 – 476] (21)	406 [359 – 459] (47)	317 [297 – 338] (127)	226 [210 – 234] (131)
B - 12 < 148 pmol/L	0 (0)	0 (0)	2 (2)	15 (20)
Folate (nmol/L)	26.2 [23.4 – 29.0] (50)	21.9 [18.8 – 25.5] (55)	40.0 [37.2 – 43.0] (126)	39.5 [36.9 – 42.3] (130)
IL-6 (pg/mL)[2]	1.5 [1.3 – 1.7][a] (71)	5.2 [3.8 – 6.9] (47)	1.1 [0.9 – 1.5][a] (144)	4.1 [3.4 – 4.9] (201)
CRP (mg/L)[2]	4.5 [3.6 – 5.6] (71)	4.4 [3.2 – 6.0] (59)	3.8 [3.1 – 4.7] (113)	5.7 [3.3 – 9.7] (25)

Data are presented as mean ± SD (*n*), Geometric mean [95% CI] (*n*) for transformed variables or percentage (*n*). ([a]) indicates significant difference between mid-gestation and delivery within cohorts and the superscript ([*])indicates significant difference between the adolescent and multiples cohort ($p < 0.05$).

Maternal Erythroferrone and Hormone Concentrations During Pregnancy

Maternal ERFE, EPO and hepcidin values and the mean concentration ratios between EPO (E), hepcidin (H) and ERFE (ER) are presented in **Table 2.3**. Ratios between these regulatory hormones were explored to capture adaptive responses to iron status (H) and erythropoietic drive and hypoxia (E and ER). 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 mid-gestation or at delivery. In the entire cohort, the geometric mean ERFE concentration did not significantly differ between the mid-gestation or delivery timepoints, and ERFE averaged 0.48 ng/mL [95%CI; 0.37, 0.61 ng/mL] at mid-gestation (26 ± 4 weeks) and 0.48 ng/mL [95%CI; 0.39, 0.60 ng/mL] at delivery (39 ± 3 weeks). At mid-gestation 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 ($p = 0.3$).

Table 2.3: Serum Iron Regulatory Hormone Concentration and Their Ratios During Pregnancy

	Multiples Cohort		Adolescent Cohort	
Variable	**Mid-gestation**	**Delivery**	**Mid-gestation**	**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, 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)
% undetectable	23$^{\#*}$ (17)	3 (2)	5 (7)	10 (20)
H/EPO	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	2.3 [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)

Data are presented as geometric mean [95% CI] (*n*) for transformed variables or percentage (*n*). $^{\#}$denotes a significant difference between mid-gestation to delivery within a cohort, *denotes a significant difference compared to the adolescent cohort ($p < 0.05$). Differences between cohorts hepcidin, H/EPO, H/ERFE concentrations were not assessed as hepcidin was measured with different assays between cohorts. Hepcidin LOD for the adolescent cohort was 5 ng/mL and for the multiples 0.39 ng/mL.

Erythroferrone's Association with Hormones and Iron Status Biomarkers

In the combined cohort, ERFE concentrations at mid-gestation were significantly elevated in women with depleted iron stores; ($p < 0.01$). A similar finding was evident at delivery (TBI < 0, $p = 0.01$; 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 ($p = 0.05$, $n = 54$).

In the combined study population at mid-gestation, ERFE concentrations were significantly higher in anemic women ($n = 27$) compared to non-anemic women ($n = 155$) ($p < 0.01$). However, at delivery differences in ERFE between anemic ($n = 52$) and non-anemic 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 **Table 2.4**. In the multiples cohort, ERFE was significantly inversely associated with Hb (~25 weeks $p = 0.03$, ~35 weeks $p = 0.002$) and hematocrit (~ 25 weeks $p = 0.05$, ~35 weeks $p = 0.01$) and thus in this cohort, ERFE was significantly higher in anemic women at both mid-gestation ($p = 0.001$, $n = 71$) and at delivery ($p < 0.01$, $n = 49$). In contrast, in the adolescent cohort there were no significant relationships between ERFE and Hb (~26 weeks $p = 0.8$; ~39 weeks $p = 0.3$) or hematocrit (~ 26 weeks $p = 0.6$; ~ 39 weeks $p = 0.26$) and ERFE concentrations did not significantly differ between anemic and non-anemic adolescents at mid-gestation ($p = 0.2$, $n = 111$) or delivery ($p = 0.7$, $n = 174$). Similar to the relationship between ERFE and Hb, the relationship between EPO and Hb was stronger in the multiples cohort (mid-gestation: multiples $\beta = -0.25$, $p < 0.001$, adolescent $\beta = -0.21$, $p < 0.001$; delivery: multiples $\beta = -0.31$, $p < 0.001$, adolescents β

= -0.21, $p < 0.001$). Additionally, in the adolescent cohort, ERFE was positively associated with IL-6 concentrations ($p < 0.01$ mid-gestation; $p = 0.05$ delivery) whereas no significant relationship between IL-6 and ERFE was observed in the multiples cohort at either mid-gestation ($p = 0.8$) or delivery ($p = 0.9$).

Erythroferrone was significantly positively associated with EPO at mid-gestation and delivery in the group as a whole (**Table 2.4**). When evaluated within each cohort, the relationship between EPO and ERFE was only significant in the multiples cohort (**Table 2.4, Figure 2.3**). Although ERFE is known to decrease hepatic hepcidin expression, there were no significant associations between ERFE and hepcidin at either mid-gestation ($p = 0.30$) 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 relationships between EPO and hepcidin at mid-gestation and delivery were explored using mediation analysis. In the combined and individual cohorts, ERFE did not mediate the relationship observed between EPO and hepcidin at mid-gestation or delivery (**Figure 2.4**). Adjusting for inflammation (CRP or IL-6), ppBMI, or Hb and SF did not alter significance of any of the relationships evaluated

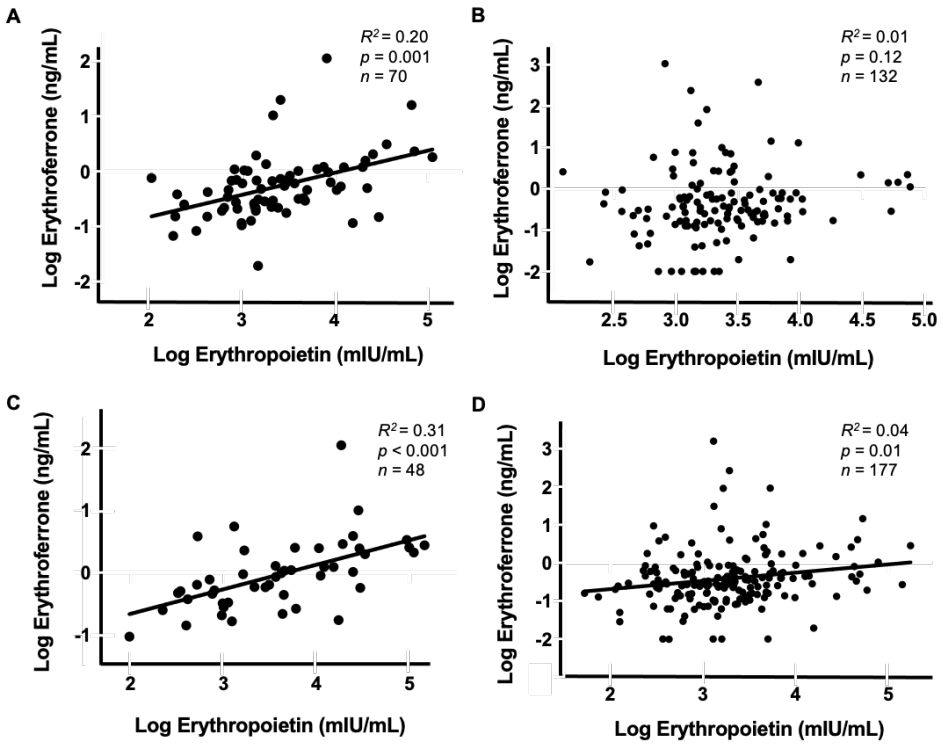


Figure 2.3: Correlations Between Erythroferrone and Erythropoietin
Bivariate correlations between EPO and ERFE in the multiples cohort at mid-gestation (A) and delivery (C). Bivariate correlations between EPO and ERFE in the adolescent cohort at mid-gestation (B) and delivery (D). EPO, erythropoietin; ERFE, erythroferrone

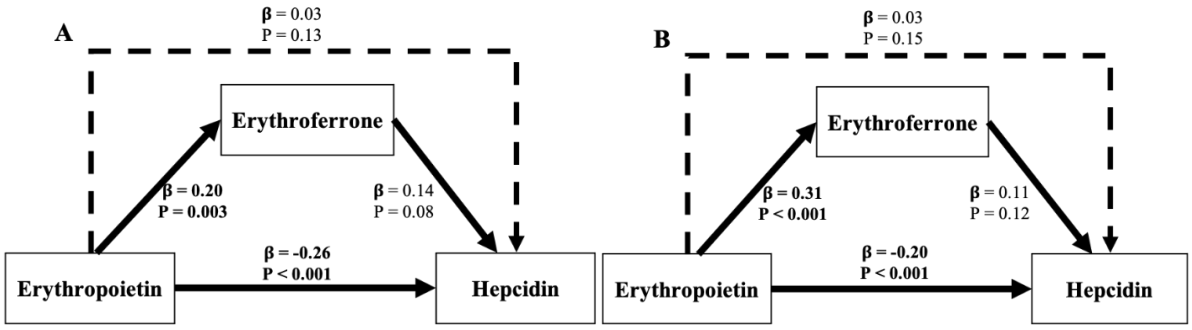


Figure 2.4: Mediation Models for Erythroferrone, Erythropoietin and Hepcidin
Mediations models were utilized to assess direct associations (solid lines) and indirect associations (dashed line) between these regulatory hormones at mid-gestation (A) and delivery (B). Bolded coefficients and p-values indicate significant effects ($p < 0.05$).

Table 2.4: Bivariate Correlations Between Erythroferrone and Iron Status

	Whole Population	Multiples Cohort	Adolescent Cohort
		Mid-gestation	
Hemoglobin	-0.08 (182)	-0.26* (71)	0.02 (111)
Hematocrit	-0.05 (184)	-0.23* (71)	0.04 (113)
Soluble Transferrin Receptor	0.38*** (208)	0.48*** (71)	0.36*** (137)
Total Body Iron	-0.27*** (208)	-0.38** (71)	-0.24* (137)
Erythropoietin	0.21** (202)	0.45*** (70)	0.12 (132)
Hepcidin/ Erythropoietin	-	-0.36* 0.003 (70)	-0.02 (131)
Interleukin-6	0.13 (205)	-0.04 (70)	0.45*** (135)
		Delivery	
Hemoglobin	-0.02 (223)	-0.43** (49)	0.08 (174)
Hematocrit	0.01 (216)	-0.37* (49)	0.09 (167)
Soluble Transferrin Receptor	0.23*** (231)	0.42** (49)	0.19* (182)
Total Body Iron	-0.17* (229)	-0.24 (49)	-0.16* (180)
Erythropoietin	0.29*** (225)	0.56*** (48)	0.19* (177)
Hepcidin/ Erythropoietin	-	-0.37* (47)	-0.15* (174)
Interleukin-6	0.14* (222)	0.02 (46)	0.15* (176)

Indicators

Pearson bivariate correlations between ERFE and iron status indicator (*n*). (*) indicates $p < 0.05$; (**) indicates $p < 0.01$, (***) indicates $p < 0.001$.

Determinants of Erythroferrone

Using all available biomarker data, an integrative model was developed to capture determinants of the variance in ERFE at both mid-gestation and delivery (**Figure 2.4**). 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 ($s\beta = 0.31$, $p < 0.001$, $n = 231$), 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 (**Table 2.5**). Consistent with the greater ERFE variability evident in the adolescent cohort, < 30 % of variance in ERFE was captured by measured variables compared to 40-50% in the multiples cohort.

Longitudinal Changes in Erythroferrone

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 – 42 weeks 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 ($\beta = 0.056$, $p = 0.06$, $n = 19$) compared to that observed in the women carrying twins ($\beta = 0.012$, $p = 0.06$, $n = 19$).

Table 2.5 Determinants of Maternal Erythroferrone

	Mid-gestation						Delivery					
	Combined		Multiples		Adolescents		Combined		Multiples		Adolescents	
	sβ	*p*	sβ	*p*	sβ	*p*	sβ	*p*	sβ	*p*	sβ	*p*
EPO			0.32	0.02					0.34	<0.001	0.25	0.004
Hb									-0.11	0.032	0.24	0.004
sTfR	0.38	< 0.001	0.45	0.003	0.41	<0.001	0.36	< 0.001			0.22	0.005
Hepcidin			0.18	0.11								
IL-6					0.32	<0.001	0.14	0.02			0.18	0.02
STI,					-0.14	0.07						
Twins/Triplets									-0.27	0.001		
Variance explained	15%		37%		30%		18%		50%		14%	

Multiple regression was undertaken to identify the strongest determinants of maternal ERFE concentrations. Data are presented as standardized β-coefficients and p-value. Blank cells indicate that the iron status indicator, hormone or characteristic was not a strong determinant of ERFE and was removed from the model. The combined cohort models controlled for study population.

Table 2.6: Serum Erythroferrone Quartiles

ERFE, ng/mL	Quartile 1	Quartile 2	Quartile 3	Quartile 4
		Mid-gestation		
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)

Data presented as mean ± SD (n*)*. *Indicates a significant difference ($p < 0.05$) between cohorts within each quartile.

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 (**Figure 2.5**). This may have been impacted by significantly greater variance in ERFE found in the adolescent cohort at both mid-gestation (Bartletts $p < 0.001$) and delivery (Bartletts $p = 0.003$). This broader range of ERFE concentrations observed in the adolescent cohort at each gestational timepoint sampled can be visualized by comparing quartiles of ERFE concentrations between cohorts (**Table 2.6**). 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, mid-gestation ERFE concentrations were significantly positively associated with ERFE concentrations at delivery ($R2 = 0.74$, $p < 0.001$, $n = 148$).

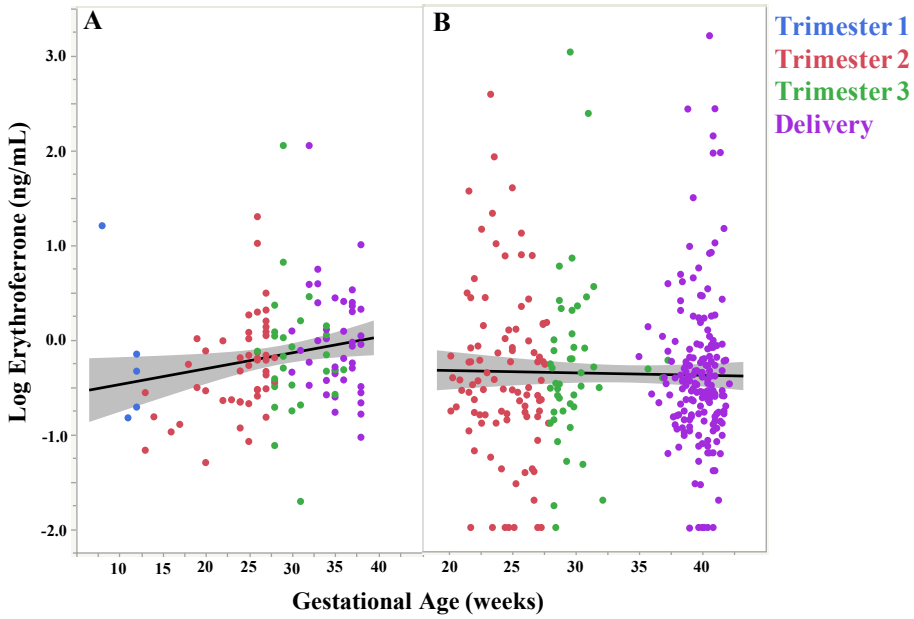


Figure 2.5: Longitudinal Change in Erythroferrone Across Pregnancy
(A) Longitudinal change in erythroferrone in the multiples cohort. (B) Longitudinal change in erythroferrone in the adolescents cohort. Different colors reflect the stage of gestation evaluated.

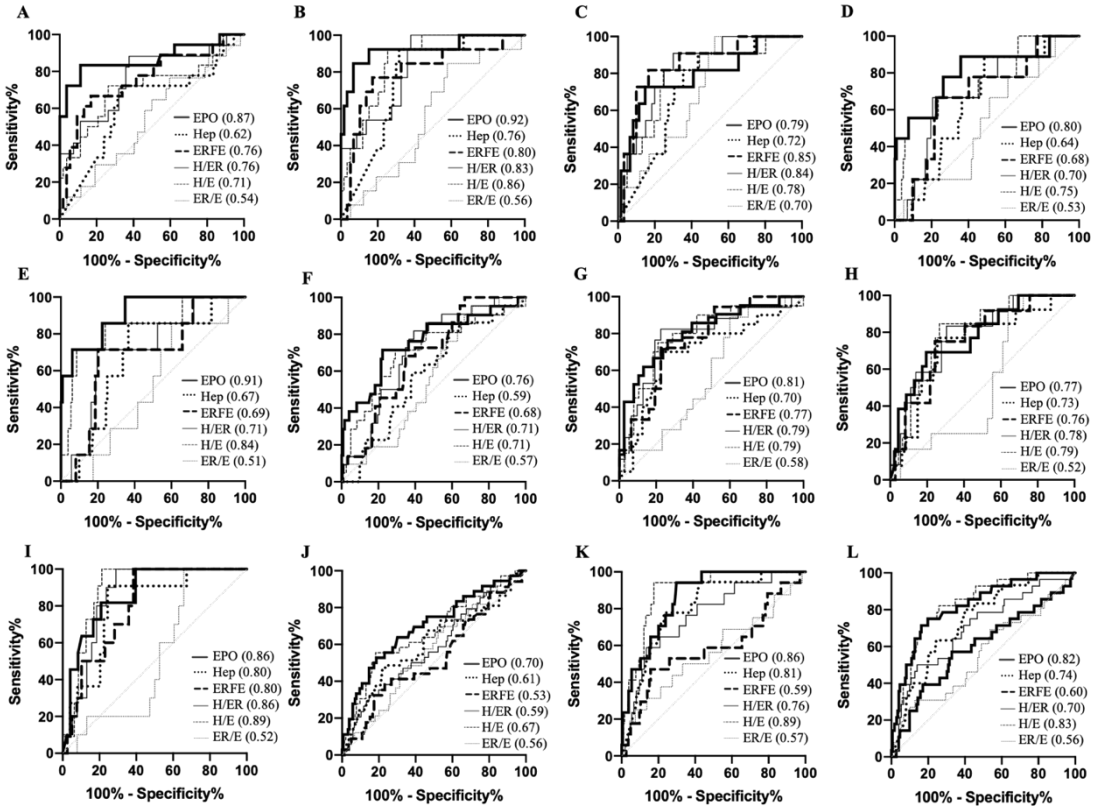
Regulatory Hormone Ratios and Predictive Ability for Identifying ID and Anemia

Given the known regulatory interrelationships between EPO, hepcidin (H) 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 TfR as they are not impacted by inflammation. At mid-gestation and delivery, the H/ERFE ratio explained more variance in EPO ($R^2 = 0.17$, $p < 0.001$; $R^2 = 0.23$, $p < 0.001$ respectively) than H ($R^2 = 0.10$; $R^2 = 0.09$) or ERFE ($R^2 = 0.10$; R2 = 0.15) alone. The H/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 to either H/ERFE, ERFE/E, EPO, ERFE or H.

To further explore the predictive ability of the iron biomarkers evaluated, multiple ROC's were generated, and the AUC were compared to explore ability of each iron regulatory hormone and hormone ratio in identifying women with anemia, IDA or depleted iron stores (**Figure 2.6**). At mid-gestation and delivery, EPO performed best in the combined cohort (mid-gestation 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 mid-gestation 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 cut-offs generated to identify women with anemia or depleted iron stores were higher in the multiples

cohort than observed in the adolescent cohort. The H/EPO ratio performed the best at identifying women with IDA at mid-gestation and delivery and the AUC for H/EPO ratio was similar to EPO for anemia and IDA.

Figure 2.6: ROC and AUC for Anemia, Iron Deficiency Anemia and Depleted Iron Stores During Pregnancy



ROC curves for identifying (A) anemia in women carrying multiple fetuses at mid-gestation, (B) iron deficient anemia in women carrying multiple fetuses at mid-gestation, (C) depleted iron stores in women carrying multiple fetuses at mid-gestation, (D) anemia in pregnant adolescents at mid-gestation, (E) iron deficient anemia in pregnant adolescents at mid-gestation, (F) depleted iron stores in pregnant adolescents at mid-gestation, (G) anemia in women carrying multiple fetuses at delivery, (H) iron deficient 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 deficient anemia in pregnant adolescents at delivery, (L) depleted iron stores in pregnant adolescents at delivery. Value next to the hormone or hormone ratio indicates the area under the curve (AUC). AUC, area under the curve; EPO, Erythropoietin; ERFE, Erythroferrone; H, Hepcidin; ROC, receiver operating characteristic curve

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 two 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 between hepcidin/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 mid-gestation and at delivery. Only two published studies to date have measured ERFE using the same assay in healthy non-pregnant 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 years) reported mean ERFE concentrations of 1.0 ± 1.13 ng/mL in non-ID elite athletes (n = 35) and 3.5 ± 5.1 (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) to those observed in these pregnant women. In sum, erythroferrone 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).

Erythropoietin 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 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 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 adult men and non-pregnant women (50, 53, 55).

Relationships 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 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 biologic 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 non-pregnant women and men (age 50 - 80y), 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 mid-gestation. 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 relationship 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 a 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 three 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: mid-gestation AUC 0.77; delivery AUC 0.84). There are no other published data using a ROC approach to evaluate relationships 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 slower AUC for this measure (AUC: 2nd trimester 0.744; 3rd trimester 0.715)(69). Additionally, in the women carrying multiples, the ROC curve

for EPO that was developed to identify risk of anemia demonstrated a sharp increase in sensitivity while maintaining high specificity at mid-gestation but not at delivery. This suggests that this indicator may have enhanced diagnostic ability in mid-pregnancy, 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 to single hormone measures. In the current study, the ratio between hepcidin/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/ERFE ratio have been published (70-72). In hemodialysis patients the hepcidin/ERFE ratio was best able to predict increases in Hb concentrations after ferric citrate hydrate administration (71). Another study in thalassemic patients found the hepcidin/ERFE ratio was significantly lower in thalassemic patients compared to normal controls (70), and Wei et.al found that the ratio between hepcidin/ERFE best predicted risk of spontaneous abortion (72).

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 two different assays

between the cohorts. This makes interpretation of the hormone ratio cut-off's difficult to interpret when comparing between studies.

Maternal ERFE was associated with erythropoietic demand during pregnancy but it was not significantly associated with hepcidin. Relationships between ERFE and iron status indicators significantly differed in adolescent pregnancies when compared to 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/EPO were best able to identify women at increased risk of anemia, ID or IDA compared to other iron regulatory hormones and ratios. Of all measures examined, the hepcidin/EPO 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.

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CHAPTER 3

IRON ABSORPTION DURING PREGNANCY IS UNDERESTIMATED WHEN IRON UTILIZATION BY THE PLACNETA AND FETUS ARE IGNORED*

*Delaney KM, Guillet R, Pressman EK, Caulfield LE, Zavaleta N, Abrams SA, O'Brien KO. Iron absorption during pregnancy is underestimated when iron utilization by the placenta and fetus is ignored. Am J Clin Nutr 2020. doi: 10.1093/ajcn/nqaa155.

Abstract

Background: Maternal iron (Fe) absorption during pregnancy can be evaluated using red blood cell (RBC) incorporation of orally administered stable Fe isotope. This approach underestimates true maternal absorption of Fe as it does not account for absorbed Fe that is transferred to the fetus or retained within the placenta.

Objective: Our objective was to re-evaluate maternal Fe absorption after factoring in these losses and identify factors associated with Fe partitioning between the maternal, neonatal and placental compartments.

Design: This study utilized data from stable Fe isotope studies carried out in 68 women during the third trimester of pregnancy. Fe status indicators and stable Fe isotopic enrichment were measured in maternal blood, umbilical cord blood and placental tissue when available. Factors associated with Fe isotope partitioning between the maternal, neonatal and placental compartments were identified.

Results: On average, true maternal absorption of Fe increased by 10% (from 19% to 21%) after accounting for absorbed Fe present in the newborn ($p < 0.001$), and further increased by 7%, (from 39% to 42%, $p < 0.001$) after accounting for Fe retained within the placenta. On average, 2% of recovered tracer was present in the placenta and 6% was found in the newborn. Net transfer of Fe to the neonate was higher in women with lower total body Fe ($s\beta = -0.48$, $p < 0.01$) and lower maternal hepcidin ($s\beta = -0.66$, $p < 0.01$). In women carrying multiple fetuses, neonatal hepcidin explained a significant amount of observed variance in net placental transfer of absorbed Fe ($R = 0.95$, $p = 0.03$).

Conclusion: RBC Fe incorporation of an orally ingested tracer underestimated true maternal Fe absorption. The degree of underestimation was greatest in women with low body iron. Maternal hepcidin was inversely associated with maternal RBC Fe utilization, whereas neonatal hepcidin explained variance in net transfer of Fe to the neonatal compartment.

Introduction

Maternal iron (Fe) deficiency anemia is associated with a higher incidence of preterm birth, low birth weight (LBW), intra-uterine growth restriction (1-3), and an adequate Fe supply is essential for normal fetal brain development (4). To prevent anemia, The Institute of Medicine recommends universal Fe supplementation for all pregnant women, regardless of maternal age or the number of fetuses carried (5). The United States Preventative Services Task Force has concluded that there is insufficient evidence to document the benefits and/or risks of universal Fe supplementation throughout pregnancy (6, 7). Determination of risks and benefits of universal Fe supplementation is constrained by insufficient knowledge regarding gestational adaptions in Fe physiology and lack of guidelines for the interpretation of Fe status indicators as a function of gestational stage (6).

Iron balance is largely controlled at the enterocyte by the hormone hepcidin. Iron absorption must be tightly regulated as humans have no physiologic means of excreting excess Fe. During pregnancy, Fe requirements increase to support increased erythropoiesis, given plasma volume expansion, as well as fetal/placental Fe needs. Early Fe radioisotope studies (8, 9), and subsequent stable Fe isotope studies (10-12) have provided key data on maternal Fe utilization across gestation. These studies quantified maternal Fe absorption based on the amount of orally administered Fe tracer that was incorporated into maternal red blood cells (RBC's), assuming a fixed fraction of absorbed tracer is incorporated into RBC. If a second Fe isotope is administered intravenously, the fraction of Fe incorporated into maternal RBC can be directly measured and used to improve estimates of maternal Fe absorption (8, 12-14).

In non-pregnant populations this approach likely captures the majority of absorbed Fe, but in pregnant women this methodology fails to account for maternally absorbed Fe that is transferred to the fetus or retained within the placenta, and thus underestimates true maternal Fe absorption. At this time the degree of underestimation, as well as the factors that impact relative utilization of absorbed Fe between the maternal, fetal or placental compartments are unknown.

A more accurate understanding of Fe utilization by the mother, fetus and placenta is needed. To address this issue, we pooled existing and new data from stable Fe isotope studies conducted in maternal-neonatal dyads to obtain novel data on relative maternal, neonatal and placental Fe utilization. The primary objective of this study was to obtain a more accurate estimation of true maternal Fe absorption during pregnancy. A secondary objective was to evaluate the impact of maternal and neonatal Fe status and Fe regulatory hormones on the observed variability in partitioning of absorbed Fe between the mother, placenta and fetus.

Methods

Participants

Stable Fe isotope studies were undertaken in a total of 68 women studied during the third trimester of pregnancy. The population of 68 women included 63 women carrying singletons (10, 14), of which 43 women (age 18 – 30 y) were recruited from the community of Villa El Salvador, Peru (studied between 1995 and 1997) and 20 women (age 16 – 32 y) were recruited from Rochester, NY (studied between 2008 and 2009) (**Figure 3.1**). Data on Fe transfer to the fetus in these women have been

published (15, 16). A third data set was comprised of unpublished data from 5 women carrying multiple fetuses (n = 4 carrying twins, n = 1 carrying triplets) who participated in Fe absorption studies in Rochester, NY between 2013 and 2014. All women were healthy with no preexisting conditions known to be associated with alterations in Fe physiology. Informed consent was obtained from all participants. The studies undertaken in Rochester, NY were approved by the Institutional Review Boards of the University of Rochester and Cornell University, and the study undertaken in Peru was approved by the Instituto de Investigación Nutricional (IIN) and the Committee on Human Research at The Johns Hopkins School of Hygiene and Public Health.

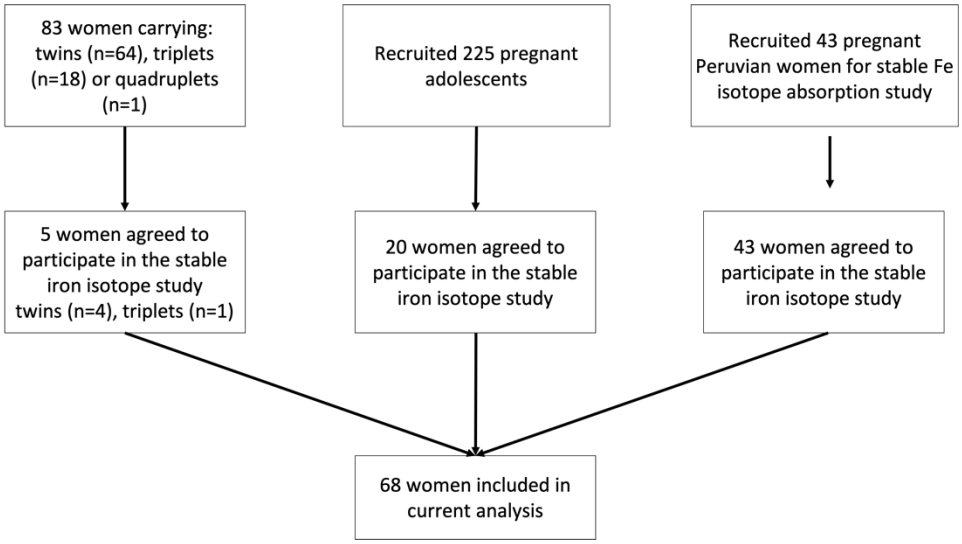

Figure 3.1 Flow chart of mothers included

Iron isotope preparation

Oral stable Fe isotope for all studies was purchased as the metal (^{57}Fe at 94.67% – 94.69%) and converted into ferrous sulfate following published procedures (17). Metal tracer was converted to ferrous sulfate and administered orally as an aqueous solution. In the Peru study the ferrous sulfate was administered without

ascorbic acid and in the Rochester studies the ferrous sulfate solution contained ascorbic acid in a 2:1 molar ratio of ascorbic acid to Fe. All stable Fe isotopic analyses were completed using a magnetic sector thermal ionization mass spectrometer (MAT 261; Finnigan, Bremen, Germany or Triton TI (Thermo Fisher Scientific, Madison, WI)

Study Design and Isotope Dosing

The study design is presented in **Figure 3.2**. Fasting women received an oral dose of ^{57}Fe averaging 9.32 ± 1.03 mg (ranging from 6.08 – 10.22 mg). Two-thirds of the Peruvian women also consumed an additional 50 mg of supplemental Fe (as ferrous sulfate) with the tracer because the intent of the study was to evaluate Fe absorption from a typical supplemental dose of Fe used in Peru at the time this study was undertaken. All women remained fasting for 1.5 hours post dosing and a maternal blood sample was obtained two-weeks post-dosing to measure Fe isotope incorporation into maternal red blood cells. At delivery, an additional sample of maternal blood and 5 – 15 mL of umbilical cord blood was collected to assess isotope incorporation into neonatal RBC's to quantify net transfer of Fe tracer to the fetus. Maternal and umbilical cord serum was isolated and stored at -80^{o}C until utilized to evaluate Fe status indicators.

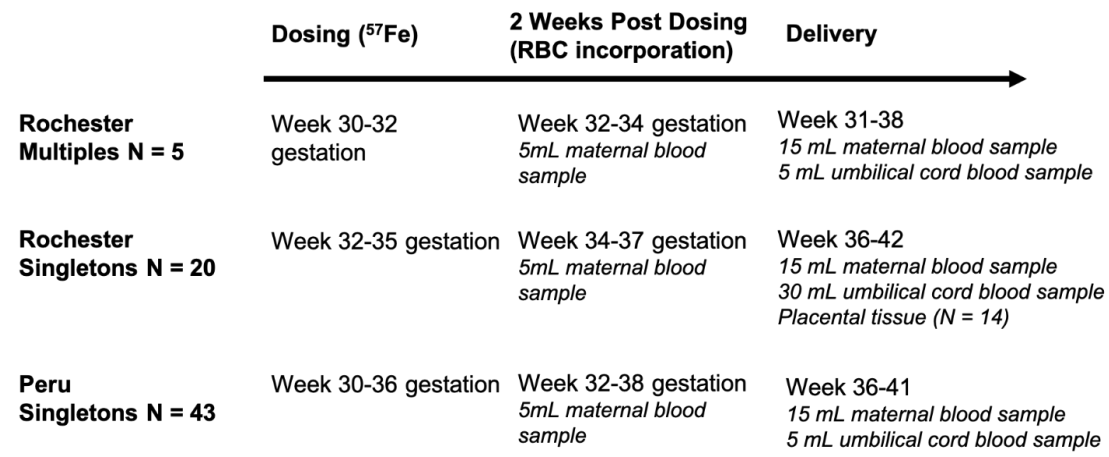

Figure 3.2: Study Timeline Study design and timing of Fe isotope dosing and sample collections in each cohort.

Biochemical assessment of iron status indicators

Within the Peru cohort, hemoglobin was measured using the cyanomethemoglobin method whereas hemoglobin (Hb) concentrations were measured using a Cell-Dyn 4000 hematology analyzer (Abbott diagnostics; Santa Clara, CA) in the Rochester cohorts. Maternal anemia in late gestation was defined as a hemoglobin concentration less than 11.0 g/dL (18) and neonatal anemia was defined as cord hemoglobin concentration less than 13.0 g/dL (19). In the Rochester cohorts, serum ferritin and serum transferrin receptor (sTfR) were measured by ELISA (Ramco Laboratories; Stafford, TX) as previously described (20, 21). In the Peru cohort, serum transferrin receptor was measured by Quantikine ELISA (R&D Systems; Minneapolis, MN) and serum ferritin was measured by ELISA (DAKO; Santa Barbara, CA). Although commercial TfR kits are not standardized, the strong correlation between R&D systems method and Ramco (22) has previously been used to justify use of TfR data from both assays to evaluate total body iron as described by Cook et al. (23, 24).

Total body iron (TBI) in all women was calculated with the following equation: TBI (mg/kg) = 2[log (serum TfR/SF) – 2.8229]/ 0.1207. The use of the TBI estimate has been documented in pregnant women (24). In the Rochester cohorts, folate, and vitamin B-12 were analyzed by immunoassay (Siemens Immulite 2000; Erlangen, Germany), and in the Peru cohort they were measured by radioimmunoassay (Diagnostic Products Corporation Immulite 2500; Los Angeles, CA). Folate deficiency was defined when folate was < 6.8 nmol/L and vitamin B12 deficiency was defined when concentrations were < 148 pmol/mL (25, 26). Hepcidin in the multiples cohort was measured using an enzyme immunoassay kit (Bachem) [Range: 0 - 25 ng/mL] or by ELISA in the Rochester singletons cohort (Intrinsic Lifesciences; La Jolla Ca) [Range: 2.5 – 1000 ng/mL]. Erythropoietin (EPO) was measured in both Rochester cohorts by immunoassay system (Siemens Immulite1000; Erlangen, Germany). In statistical analyses, cohort was controlled for to account for possible differences in assay methodology. When placental samples were obtained, the Fe content of the placenta was measured by Perkin Elmer Axial Field Technology DRC II ICP/MS (Perkin-Elmer, Norwalk CT) as previously reported (27).

Iron isolation from biological samples and mass spectrometric analysis

Whole blood (1 mL) and ~1 g of placental tissue (a mixture of tissue was pooled from multiple sites sampled across the syncytiotrophoblast) were digested with 5-15 mL Ultrex nitric acid (JT Baker, Phillipsburg, NJ) and evaporated to dryness. The dried residue was reconstituted in 2 – 4 mL 6N Ultrex hydrochloric acid (JT Baker; Phillipsburg, NJ), extracted using anion exchange chromatography and the eluate was heated until dry. The Fe residue was reconstituted in 30 µL nitric acid as

previously described (14). Extracted Fe samples were loaded onto rhenium filaments and isotopic ratios ($^{57/56}$Fe, $^{58/56}$Fe, and $^{54/56}$Fe) were measured using magnetic sector thermal ionization mass spectrometry as previously described (Triton, ThermoFisher; Waltham MA or MAT 261; Finnigan, Bremen, Germany) (14, 15, 28).

Approaches used to evaluate maternal iron absorption and iron partitioning

Stable Fe isotope enrichment in maternal blood, placental tissue and umbilical cord blood was expressed as a delta percent excess (Δ% excess), which is the degree to which the measured $^{57/56}$Fe ratio was increased over the corresponding natural abundance $^{57/56}$Fe ratio. In the maternal compartment, the net ^{57}Fe recovered was also determined using the $^{57/56}$Fe enrichment of maternal RBC's 14 days post-dosing, maternal weight, hemoglobin concentration, maternal blood volume (70 mL/kg)(14), and the Fe content of hemoglobin (3.47 g/kg)(29), and assuming 80% of absorbed Fe was incorporated into maternal RBC's.

The net quantity of ^{57}Fe recovered (mg) in the neonate was estimated at birth using the same approach and an estimated newborn blood volume of 80 mL/kg (15). In the newborn, it was assumed that 80% of absorbed isotope was incorporated into RBC's (13, 30), based on autopsy data showing that 75-80% of the total Fe present in the newborn is found within their RBC's (31, 32). Net ^{57}Fe recovered in placental tissue was calculated using the untrimmed placental wet weight, $^{57/56}$Fe enrichment, and the Fe content of the placenta. To account for excess weight contributed by the placental membranes and umbilical cord, a factor of 16% was removed from the untrimmed weight (33). Iron recovered in each compartment was expressed as either

the net mg quantity of ^{57}Fe recovered or as the net mg of ^{57}Fe recovered per kg of placental, or neonatal birth weight.

Three different approaches were utilized to evaluate maternal Fe absorption. The first approach followed standard methodology and evaluated ^{57}Fe incorporation into maternal RBC's 14 days post dosing (M absorption, n = 66). The second approach also factored in the total amount of ^{57}Fe recovered in neonatal RBC's at birth (M+N absorption, n = 60). The third approach included the total amount of ^{57}Fe recovered in placental tissue when available (M+N+P absorption; n = 14).

Iron partitioning between the maternal, neonatal or placental compartment was calculated as the net ^{57}Fe recovered (mg) in each compartment as a fraction of the total 57Fe recovered (mg) in all three compartments. The variable approaches utilized are depicted in **Figure 3.3**. To control for the slight differences in dose of stable isotope consumed by each women, all values were dose adjusted to a mean dose of 8.35 mg (9). Significant findings remained significant if non-adjusted doses were utilized.

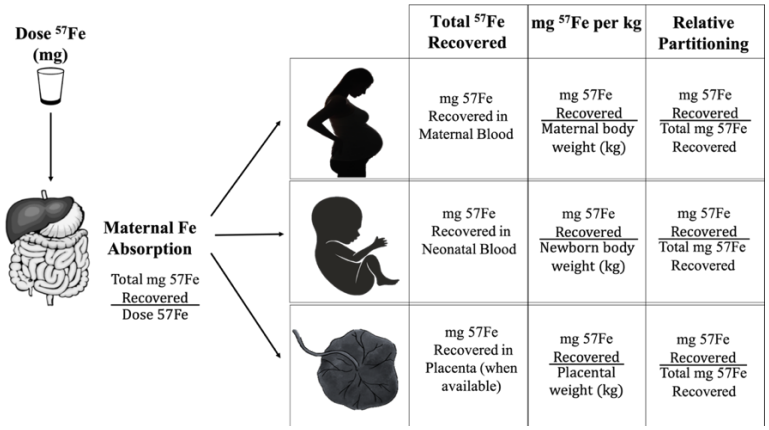

Figure 3.3: Calculations of Partitioning of Iron Between Compartments
Diagram depicting calculations used to determine maternal absorption, total Fe recovered, mg Fe per kg of weight and partitioning of Fe between maternal, neonatal and placental compartments. Maternal absorption was calculated using RBC Fe incorporation of ^{57}Fe 2 weeks post-dosing. Stable iron enrichment in the neonatal and placental compartments was calculated using samples collected at birth.

Statistical analysis

All statistical analyses were completed using JMP 13.0 (SAS Institute; Cary, NC). Non-normally distributed values were transformed prior to statistical analysis. Possible differences by cohort was assessed by ANOVA, chi-square or Wilcoxon rank sums test. Multiple linear regression analysis was used to assess relationships between Fe absorption and partitioning with Fe status indicators and hormones while controlling for study and with Maternal ID as a random effect to control for neonates in the multiples cohort being born from the same mother. Significance was considered as $p < 0.05$. The sβ abbreviation indicates standardized β reported for individual correlations between Fe regulatory hormones and Fe recovery that are controlled for study.

Results

Subject characteristics

Maternal and neonatal characteristics are presented in **Table 3.1**. Women carrying multiple fetuses were significantly older than women carrying singletons ($p < 0.001$), and maternal body mass index (BMI) at entry into the study differed significantly by cohort ($p < 0.001$). None of the women in this partitioning study developed pre-eclampsia. Twenty six percent of newborns were small for gestational age (SGA) (n = 13/41 Peru and n = 4/31 Rochester). Although the majority of the neonates born to women carrying multiples were LBW, none were classified as SGA. In the multiples cohort, discordant growth was not evident among neonates based on the American College of Obstetricians and Gynecologists threshold of a 15-25% difference in birth weight between siblings (34).

Maternal Fe status indicators at delivery are presented in **Table 3.2**. At delivery, the prevalence of maternal anemia was 31% and did not significantly differ between the three cohorts ($p = 0.83$). None of the women from the Rochester cohorts were folate deficient (serum folate < 6.8 nmol/L), however 21% of women in the Rochester singletons cohort were vitamin B12 deficient (serum vitamin B12 < 148 pmol/L). In the Peru cohort, 11% of women were folate deficient and 37% were vitamin B12 deficient. Data on umbilical cord Fe status indicators are also presented in **Table 3.2**. In the combined cohort, the prevalence of neonatal anemia was 14%. Prevalence of neonatal anemia was significantly higher in the Rochester singletons cohort ($p < 0.01$) consistent with other findings in neonates born to adolescents and with animal data showing altered nutrient partitioning in adolescent pregnancies (21, 35). Folate status was adequate in all newborns, but 25% of all newborns studied were vitamin B12 deficient (12/24 in the Peru cohort and 1/18 in the Rochester singletons cohort).

Table 3.1: Maternal and Neonatal Characteristics

Variable	Combined	Singletons (P)	Singletons (R)	Multiples
Maternal	N = 68	N = 43	N = 20	N = 5
Age (y)	22.73 ± 5.00	23.26 ± 4.38[a]	19.55 ± 3.87[b]	31.0 ± 2.34[c]
BMI	28.95 ± 5.64	26.48 ± 2.66[b]	32.52 ± 6.67[a]	35.64 ± 7.74[a]
Neonatal	N = 74	N = 42	N = 19	N = 11
GA (wk)	38.63 ± 0.05	39.75 ± 1.00[a]	39.52 ± 1.60[a]	33.81 ± 2.52[b]
PTB (%)	20	03[a]	16[a]	82[b]
Birth Weight (g)	2970.96 ± 587.91	3104.14 ± 417.56[a]	3169.74 ± 548.25[a]	2107.00 ± 492.79[b]
LBW (%)	21	05[a]	21[b]	82[c]
SGA (%)	26	37[a]	21[b]	0[c]
Male (%)	52	44	68	45

Values are presented as mean ± SD. BMI value is from third trimester at the beginning of the study. Data were analyzed by Wilcoxon Rank Sum Test, ANOVA or Chi-Square test. Differing superscripts[a,b,c] indicate significant differences between cohorts.

Table 3.2: Maternal and Neonatal Iron Status Indicators at Delivery

	Singletons (P)	Singletons (R)	Multiples
Maternal Indicators	**N= 43**	**N= 20**	**N= 5**
Hb (g/dL)	11.92 ± 1.77	11.15 ± 1.18	11.64 ± 2.30
Anemic (%)	30	37	20
SF (ug/L)	32.49 [23.37, 45.17]	19.47 [12.19, 23.54]	27.50 [7.50, 100.93]
< 20 μg/L	33	40	67
< 12 μg/L	17	22	33
sTfR (mg/L)	3.66 [2.98, 4.49]	6.26 [5.01, 7.83]	5.53 [0.61, 49.66]
TBI (mg/kg)	3.39 ± 3.72	2.30 ± 3.03	2.89 ± 5.98
Hepcidin (ng/mL)	-	36.92 ± 58.20 [2.5, 207.1]	9.59 ± 12.89 [1.2, 24.4]
EPO (IU/L)	-	32.61 ± 23.00 [9.4, 109.5]	44.7 ± 38.24 [10, 185.7]
Folate (nmol/L)	12.42[b] [10.30, 14.97]	37.40[a] [30.20, 46.32]	20.32[ab] [8.83, 46.81]
Deficient (%)	11	0	0
Vitamin B12 (pmol/L)	178.52 ± 81.57[a]	262.21 ± 123.03[b]	343 ± 101.06[b]
Deficient (%)	37	21	0
Neonatal Indicators	**N = 42**	**N = 20**	**N = 11**
Hb (g/dL)	15.71 ± 1.89[a]	13.36 ± 3.05[b]	15.37 ± 1.13[a]
Anemic (%)	7[a]	37[b]	0[a]
SF (ug/L)	167.84 ± 77.68	202.01 ± 115.97	148.46 ± 52.83
sTfR (mg/L)	6.23 ± 1.79	8.35 ± 2.15	7.68 ± 2.55
TBI (mg/kg)	10.18 ± 2.49	9.43 ± 2.80	9.10 ± 2.61
Hepcidin (ng/mL)	-	62.08 [8.5, 95.94]	16.13 [9.58, 27.16]
EPO (IU/L)	-	28.13 [17.07, 46.35]	14.92 [10.42, 21.37]
Folate (nmol/L)	24.36 ± 8.50[b]	69.44 ± 26.61[a]	85.59 ± 23.54[a]
Deficient (%)	0	0	0
Vitamin B12 (pmol/L)	140.91[b] [110.72, 179.33]	510.88[a] [348.05, 749.89]	408.58[a] [298.94, 558.42]
Deficient (%)	50[b]	6[a]	0[a]

[1] Values are presented as mean ± SD or geometric mean [95%CI]. Data were analyzed by ANOVA or Chi-Square test to assess indicator differences between cohorts measured with the same assay. Differing superscripts[a,b,c] indicate significant differences between cohorts. Hb, hemoglobin; SF, serum ferritin; sTfR, soluble transferrin receptor; P, Peru; R, Rochester

Maternal tracer enrichment and net amount of tracer recovered in maternal RBC

The net mg of ^{57}Fe recovered in the maternal compartment significantly differed by cohort ($p < 0.001$) (**Table 3.3**). Maternal RBC ^{57}Fe incorporation (M absorption) averaged 19% [95% CI: 16%, 23%] and is presented in **Table 3.4**. Maternal TBI explained 70% of the observed variance in net ^{57}Fe recovered (mg) in maternal RBC ($s\beta = -0.19$ $p = 0.01$, $n = 60$) and M absorption ($s\beta = -0.19$, $p = 0.01$, $n = 60$). M absorption was positively associated with maternal EPO (both $s\beta = 0.04$, $p = 0.05$, $n = 21$) and negatively associated with maternal hepcidin ($s\beta = -0.48$, $p = 0.04$, $n = 21$).

Neonatal tracer enrichment and net amount of tracer recovered in neonatal umbilical cord RBC

The net mg of ^{57}Fe recovered in the neonatal compartment significantly differed by cohort ($p < 0.001$) (**Table 3.3**). Net ^{57}Fe recovered (mg) in the neonate was significantly higher in neonates born to anemic mothers ($p = 0.02$, $n = 60$) and in those born to women with depleted Fe stores (SF < 20 μg/L; $p < 0.001$, $n = 59$). In the combined cohort, there was no significant association between net amount of ^{57}Fe (mg) recovered in the neonatal compartment and the number of days that had elapsed between dosing and parturition (across a range of 7 - 69 days). Net ^{57}Fe recovered (mg) in the neonatal compartment was also positively associated with net ^{57}Fe recovered (mg) in the maternal compartment ($R^2 = 0.59$ $p < 0.001$, $n = 60$). The strongest determinants of net ^{57}Fe recovered (mg) in the neonatal compartment were maternal TBI ($s\beta = -0.48$, $p < 0.001$, $n = 52$) and neonatal Hb ($s\beta = 0.37$, $p < 0.001$, n

= 52), which together explained 77% of the variance in net ^{57}Fe recovered (mg) in neonatal RBC ($p < 0.001$). In women with Fe regulatory hormone data available, women with higher hepcidin concentrations transferred less Fe tracer to their neonates ($s\beta = -0.66$, $p = 0.01$, $n = 25$). Similarly, neonates with higher hepcidin concentrations at birth had a lower quantity of tracer present in neonatal RBC's at birth ($s\beta = -0.42$, $p = 0.06$, $n = 29$). Maternal EPO was positively associated with Fe transfer to the neonatal compartment ($s\beta = 0.61$, $p = 0.01$, $n = 25$). Neonates with higher EPO had significantly less Fe tracer transferred across the placenta ($s\beta = -0.44$, $p = 0.02$, $n = 29$). The only significant correlation between maternal and neonatal hormones was a positive association between maternal hepcidin and neonatal EPO ($s\beta = 0.44$, $p = 0.03$, $n = 25$).

As expected, neonates born to women carrying multiple fetuses weighed significantly less at birth and were born at a significantly earlier gestational age. To address the possible impact of variability in neonatal birth weight on the net amount of ^{57}Fe recovered, ^{57}Fe recovery data were expressed per kg of birth weight (**Table 3.3**). Significant determinants of neonatal ^{57}Fe (mg/kg) were neonatal Hb and maternal TBI, which explained 73% of the variance in this measure ($p < 0.001$). In cohorts with Fe regulatory hormone data available, net ^{57}Fe mg/kg was significantly inversely associated with maternal hepcidin ($s\beta = -0.66$, $p < 0.01$). Neonates had a significantly greater ^{57}Fe content on a mg/kg basis when maternal EPO concentrations were elevated ($s\beta = 0.46$, $p = 0.02$) or when neonatal EPO concentrations were low ($s\beta = -0.48$, $p < 0.01$). As previously mentioned, maternal and neonatal EPO were not significantly correlated ($s\beta = 0.001$, $p = 0.9$, $n = 25$).

Table 3.3: Iron Isotope Enrichment in Maternal and Neonatal Blood

	Observed Isotope Enrichment (%)	Total Net ^{57}Fe Recovered (mg)	Net ^{57}Fe Recovered (mg/kg)	Partitioning (%)
All Groups				
Mom	3.27 [2.90, 3.89]^{a} n=64	1.24 [1.21, 1.55]^{a} n=66	0.02 [0.01, 0.02] n=66	91.21 [90.12, 92.31]^{a} n=61
Baby	3.66 [3.06, 4.31]^{b} n=61	0.11 [0.10, 0.13]^{b} n=61	0.03 [0.03, 0.4]^{b} n=61	8.12 [7.11, 9.19]^{b} n=61
Total	-	1.50 [1.24, 1.80]^{c} n=61	-	-
P Singletons				
Mom	2.54 [2.13, 2.98] n=43	0.72 [0.59, 0.87]^{a} n=43	0.01 [0.01, 0.01]^{a} n=43	90.01 [88.48, 91.55]^{b} n=37
Baby	2.47 [2.05, 2.92] n=37	0.07 [0.06, 0.08]^{b} n=37	0.02 [0.02, 0.03]^{b} n=37	9.47 [8.10, 10.94]^{a} n=37
Total	-	0.81 [0.66, 0.97]^{c} n=37	-	-
R Singletons				
Mom	5.21 [4.19, 6.35]^{a} n=18	2.62 [2.19, 3.08]^{a} n=18	0.03 [0.03, 0.04] n=18	94.14 [92.94, 95.35]^{b} n=18
Baby	6.62 [5.21, 8.20]^{b} n=19	0.17 [0.12, 0.23]^{b} n=19	0.05 [0.04, 0.07] n=19	5.62 [4.52, 6.82]^{a} n=18
Total	-	2.78 [2.32, 3.29]^{c} n=18	-	-
R Multiples				
Mom	4.22 [2.70, 6.08]^{a} n=5	2.45 [1.20, 4.13]^{a} n=5	0.03 [0.02, 0.03]^{a} n=5	90.53 [87.47, 93.64]^{b} n=5
Baby Unit	4.94 [4.08, 5.87]^{b} n=5	0.21 [0.11, 0.34]^{b} n=5	0.05 [0.03, 0.06]^{b} n=5	8.32 [3.84, 14.52]^{a} n=5
Total	-	2.55 [1.84, 3.36]^{c} n=5	-	-
R Singletons with Placenta Available				
Mom	5.20 [3.93, 6.48] n=14	2.62 [2.08, 3.16] n=14	0.03 [0.02, 0.04] n=14	92.27 [90.61, 93.93] n=14
Baby	6.59 [4.95, 8.23] n=14	0.18 [0.11, 0.24] n=14	0.06 [0.04, 0.07] n=14	5.91 [4.43, 7.38] n=14
Placenta	5.91[4.58, 7.24] n=14	0.05 [0.03, 0.06] n=14	0.10 [0.07, 0.12] n=14	1.82 [1.44, 2.20] n=14
Total	-	2.82 [2.22, 3.42] n=14	-	-

Values are presented as mean [95% CI]. Differences between mom, baby and/or placenta were analyzed by ANOVA and differing superscripts^{a,b,c} indicate significant differences within cohorts between the mother, neonate and placenta. P, Peru; R, Rochester.

When the total amount of ^{57}Fe recovered in both maternal and neonatal compartments was used to calculate net M+N Fe absorption, (n = 60), the estimation of true maternal absorption increased by 10% ($p < 0.001$) i.e. from 19% (M absorption) to 21% [95% CI: 17%, 26%] (M+N absorption) (**Table 3.4**). As expected, anemic mothers (n = 21) absorbed more ^{57}Fe than non-anemic women ($p = 0.02$, n = 39), regardless of approach used (M or M+N absorption). Additionally, M+N absorption was higher in women with SF < 20 ug/L (n = 39) compared to those with SF >20 ug/L (n = 20, $p < 0.001$). Maternal TBI and neonatal Hb together explained 73% of variance found in M+N absorption ($p < 0.001$). In women with regulatory hormone data available, M+N absorption was associated with neonatal hepcidin ($s\beta = -0.70$, $p < 0.01$, n = 23), maternal EPO ($s\beta = 0.56$, $p = 0.01$, n = 21) and maternal hepcidin ($s\beta = -0.50$, $p = 0.02$, n = 21).

Placental Tracer Enrichment

In the current study, net placental Fe content averaged 39.4 mg [95% CI: 32.2 mg, 46.2 mg] or 74.3 μg/g wet weight [95% CI: 66.2 μg/g, 82.4 μg/g]. Placental Fe content did not significantly differ between mothers that did (n = 4) or did not (n = 10) have anemia ($p = 0.80$). Placental Fe content also did not significantly different between mothers with (n = 5) or without (n = 9) Fe depletion (SF < 20 μg/L) at delivery ($p = 0.92$). Placental Fe content was lower in women with (n = 9) gestational Fe depletion (based on SF < 20 μg/L), a difference that approached significance ($p = 0.14$). Maternal TBI was inversely associated with the net amount of ^{57}Fe recovered in the placental compartment (on both a mg and a mg/kg basis) and explained 40% and 36% of variance respectively (both $p = 0.02$).

Within the group of 14 women who had placental tissue available, M absorption averaged 39% [95% CI: 31%, 47%] and M+N absorption averaged 42% [95% CI: 33%, 51%]. After addition of the net amount of ^{57}Fe recovered in the placental compartment, the estimation of true maternal absorption increased by 17% ($p < 0.001$) to 42% [95% CI: 33%, 51%] (M+N+P absorption). M+N+P absorption was significantly associated with maternal Hb and TfR and neonatal SF, which together explained 70% of the variance in true maternal absorption ($p = 0.002$, $n = 14$).

Tracer enrichment in women carrying multiple fetuses

In women carrying multiple fetuses, each individual neonate accrued a significantly lower amount of tracer compared to the mean accrued by each singleton neonate (0.09 mg [95% CI: 0.07 mg, 0.13 mg] vs. 0.17 mg, $p = 0.02$). However, if the net ^{57}Fe transferred (mg) to all siblings was summed, the net amount of tracer that each women in the multiples cohort transferred to their newborns was comparable to the amount of ^{57}Fe transferred (mg) to each singleton neonate (0.21 mg vs. 0.17 mg, $p = 0.30$) (**Table 3.4**). In addition, neonates in both cohorts accrued a similar amount of ^{57}Fe when expressed per kg of birth weight (0.054 mg/kg vs. 0.045 mg/kg, $p = 0.53$).

In the multiples cohort, variability between neonates in the same uterine compartment was calculated as the largest difference observed between siblings at birth. The mean Δ% excess in this group was 5.02 ± 1.39% and the average variability between sibling was 0.62% [95% CI: 0.24%, 1.47%]. The mean net ^{57}Fe recovered (mg) in this group was 0.10 ± 0.05 mg and the average variability between sibling was 0.015 mg [95% CI: 0.002 mg, 0.027 mg]. The mean net ^{57}Fe recovered (mg) per kg birth weight in this group was 0.05 ± 0.01 mg/kg and the average variability between

siblings was 0.01 mg/kg [95% CI: 0.003 mg, 0.0096 mg]. The strongest determinant of the observed variability between all three measurements of ^{57}Fe transfer to the neonate was the variability in hepcidin between neonates (Δ% excess: R = 0.95 p = 0.03; net 57Fe (mg): R = 0.41, p = 0.60; net 57Fe (mg/kg): R = 0.47, p = 52).

Iron partitioning between compartments

Relative utilization of maternally absorbed Fe between the maternal, neonatal and placental compartments was explored. In the combined cohort, the highest percentage of recovered ^{57}Fe tracer was utilized by the maternal compartment (**Table 3.4, Figure 3).** The strongest negative determinant of relative partitioning of absorbed 57Fe to the neonatal compartment was maternal TBI (sβ = -0.31, p = 0.01, n = 64) with lower maternal TBI being associated with significantly greater transfer of maternally absorbed tracer to the fetus. Maternal TBI explained 35% of the variance in partitioning of ^{57}Fe between maternal and neonatal compartments. Partitioning of Fe to the neonatal compartment was significantly inversely associated with total ^{57}Fe recovered (mg) (sβ = -0.52 p = 0.01, n = 60). Iron partitioning to the neonatal compartment was significantly different by cohort (p = 0.001), with Rochester singletons receiving significantly less absorbed Fe. This finding did not appear to be driven by differences in maternal age as partitioning was not significantly associated with maternal age when examined as a continuous variable (p = 0.21) or categorically in pregnant adolescents (< 20 y ; n = 19) compared to those > 20 y (n = 41; p = 0.24). In the subset of women with Fe regulatory hormone data, relative partitioning of 57Fe to the neonatal compartment was significantly inversely associated with both maternal hepcidin (sβ = -0.55 p < 0.01, n = 24) and neonatal EPO (sβ = -0.52, p < 0.01, n = 22).

Partitioning of 57Fe to the neonatal compartment was not significantly associated with maternal EPO or neonatal hepcidin.

Although not statistically significant, there were negative trends between placental partitioning and both maternal vitamin B_{12} status [β 95%CI: -3.12, 0.69], maternal folate status [β 95%CI: -23.13, 0.04] and neonatal folate status [β 95%CI: -0.06, 0.01]. Total tracer partitioning to the neonatal/placental unit was inversely associated with maternal hepcidin [β 95%CI: -2.10, -0.23] and maternal TBI [β 95% CI: -1.05, -0.23].

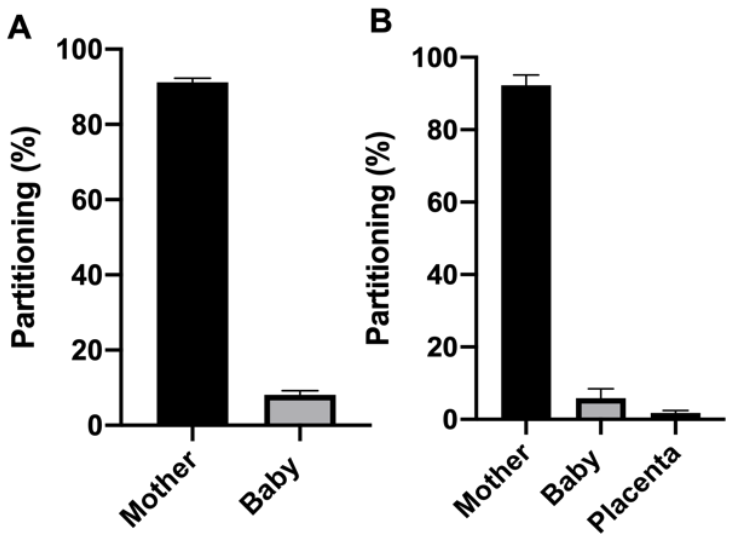

Figure 3.4: Iron Tracer Partitioning Between Mother, Newborn and Placenta
A. Partitioning of Fe between the maternal and neonatal compartment is shown for the combined cohort (N=61). The bar chart presents the % of total Fe partitioned to each compartment [95%CI]. In the multiples cohort, the baby component reflects the sum of all tracer recovered in the neonatal compartments. B. Partitioning of Fe between the maternal, neonatal and placental compartments is shown for women in the Rochester Cohort who had placental tissue available for analysis of isotopic enrichment.

Table 3.4: Percent Iron Absorption After Inclusion of ^{57}Fe Recovered From the Neonatal and Placental Compartments

Compartments Included In Analysis	Combined	Singletons (P)	Singletons (R)	Multiples
Mother	19.14 [15.45 - 23.24] N=66	10.84[a] [8.87 – 13.01] N=43	39.17[b] [32.81 – 46.10] N=18	36.61[b] [17.96 – 61.83] N=5
Mother and Neonate	21.26 [17.22 - 25.73] N=60	12.13[a] [9.93 – 14.55] N=37	41.68[b] [34.764 – 49.22] N=18	39.92[b] [20.53 – 65.70] N=5
Difference in abs	1.56 [1.29 – 1.86] N=60	1.13[b] [0.96 – 1.31] N=37	2.62[a] [1.84 – 3.41] N=18	3.26[a] [1.37 – 5.16] N=5
			N = 14	
Mother			39.19 ± 13.92	
Mother and Neonate			41.86 ± 15.16	
Mother, Neonate and Placenta			42.21 ± 15.50	
Difference in abs			3.42 ± 2.10	

Mean ± SD or geometric mean [95% CI]. Differences between absorption estimations were analyzed by ANOVA and differing superscripts[a,b,c] indicate significant differences within each cohort. Abs, absorption; P, Peru; R, Rochester

Discussion

This is the first human study to describe significantly higher maternal Fe absorption during pregnancy after accounting for the amount of absorbed tracer that was transferred to the fetus or retained within the placenta. The degree of underestimation significantly varied as a function of maternal Fe status and was greatest in women with depleted body Fe stores. Both maternal and neonatal hepcidin were associated with net transfer of absorbed Fe to the fetus. The impact of fetal hepcidin on Fe utilization was also evident among the multiple birth neonates, as in this group neonatal hepcidin concentrations captured the largest amount of variability in observed uptake of Fe between siblings.

Maternally absorbed Fe that is rapidly transferred to the neonatal/placental unit cannot readily be quantified, and therefore maternal absorption estimations used in the current dietary recommendations rely solely on estimates of Fe utilized by maternal erythropoiesis (36, 37). Pregnant women rapidly transfer orally ingested radioisotopic Fe to the fetus, as radioactivity has been detected in the infant within 40 minutes of the mother ingesting the Fe radioisotope (38). In this group of pregnant women, ~ 5% of maternally absorbed Fe was present in the newborn at birth and a slightly larger amount (2%) of maternally absorbed Fe was retained within placental tissue. As expected, inclusion of the Fe recovered within the neonate and placenta at birth resulted in a significant increase in true maternal Fe absorption. The degree of underestimation was not constant but was instead impacted by maternal Fe status, with Fe insufficient women transferring the largest fraction of absorbed Fe to the neonatal/placental compartment.

The placenta mediates nutrient transfer to the fetus and is exposed to both maternal and fetal regulatory signals. Although total placental Fe content was not significantly associated with maternal Fe status, we found that the net amount of ^{57}Fe retained in the placenta in late gestation (on both a mg and mg/kg basis) was inversely associated with maternal TBI in our population where 28% of women studied were anemic at delivery. Recent animal data found acute placental uptake of an IV dose of stable Fe isotope 6 hours post dosing did not significantly differ by the Fe status of the dam (39). To our knowledge, this is the first human study to describe human placental retention of an orally ingested Fe tracer and to assess relationships between placental retention of Fe and maternal and neonatal Fe status.

The developing fetus accumulates ~ 300 mg of Fe across the 280-day gestational period with Fe transfer being highest in the third trimester of pregnancy. In the current study the net amount of ^{57}Fe transferred to the fetus over late gestation and measured within the neonate at birth was inversely associated with maternal TBI. This association remained significant when expressed as net tracer recovered or when expressed as tracer recovered per kg of birth weight. Additionally, partitioning of absorbed Fe to the neonatal compartment was higher in women with low TBI. While studies have found maternal Fe deficiency compromises neonatal Fe stores at birth (40-43), our dynamic measures of placental Fe transfer and other studies evaluating placental Fe trafficking proteins (39, 44-47) indicate that the human placenta responds to Fe insufficiency by upregulating Fe transfer to the neonatal compartment when maternal Fe status is low.

Iron homeostasis and tissue utilization are regulated by three hormones; hepcidin, EPO and erythroferrone (ERFE). A commercial assay for ERFE was not available when these studies were undertaken, but EPO and hepcidin data were available in a subset of these women and neonates. In the current study maternal EPO concentrations were positively associated with both maternal absorption (both M and M+N absorption) and net transfer of absorbed ^{57}Fe to the neonate. Interestingly, neonatal EPO was negatively associated with net ^{57}Fe recovered in the neonate and partitioning of absorbed ^{57}Fe to the neonatal compartment. Data on determinants of neonatal EPO concentrations at birth are limited. Studies suggest that umbilical cord EPO is increased in neonates that experience hypoxic intrauterine conditions (48, 49), as EPO is inversely correlated with cord blood pH, blood gas values (50, 51) and oxygen tension (52), however some have not found significant associations between umbilical cord EPO and Hb (40, 53-56). The relationship between EPO and Fe transfer is supported by other human data showing that neonates with higher umbilical cord EPO concentrations had lower Fe status as measured by cord ferritin (57, 58).

Maternal hepcidin concentrations were inversely associated with the net amount of Fe transferred to the fetus in late gestation, consistent with other published human pregnancy data using stable Fe isotopes (11, 15). Hepcidin concentrations increase to prevent Fe overload, but this hormone is also impacted by inflammation and may be elevated in obese pregnant women (59-61) as well as in pregnant women with an increased risk of gestational diabetes (62). Given our observed association between elevated maternal hepcidin concentrations and reduced Fe transfer and partitioning of absorbed Fe to the neonatal compartment, Fe transfer to the fetus may

be compromised in these populations and further studies in these groups may be warranted.

Studies in women carrying multiple fetuses allowed us to evaluate the impact of neonatal Fe status indicators on variability seen in ^{57}Fe accrual between siblings. Cord hepcidin concentrations were a significant determinant of net ^{57}Fe transfer to the neonate and of the observed ^{57}Fe variability between siblings at birth. This finding is consistent with prior data in a larger cohort of women carrying multiples in which cord hepcidin concentrations explained ~60% of the observed intrauterine variability in Fe status (63). Together these new tracer data and our prior indicator data in these women carrying multiples suggest that the fetus has some capacity to regulate its own Fe status. This conclusion differs from recent animal studies in which hepcidin knock out fetuses exhibited no deficits in fetal Fe status when compared to wild type animals (39).

A main strength of this study was the ability to assess Fe isotopic enrichment in the maternal, neonatal and placental compartments and identify factors associated with differential Fe partitioning between these compartments. However, there are limitations. The current study included only a small number of women in the multiples group. Women participating in the tracer study were recruited from a larger cohort where 35% of women gave birth to infants who exhibited discordant growth (64). As these women did not give birth to infants with growth discordance, they may not be representative of all women carrying multiple fetuses. Second, we were only able to evaluate maternal and neonatal isotopic enrichment within the RBC compartment and cannot capture Fe tracer that was sequestered within other maternal or neonatal

tissues. This may be less of a concern in neonates as they prioritize Fe in support of RBC Fe demands (31).

Values of maternal Fe absorption during pregnancy are significantly higher when inclusion of maternally absorbed Fe that is recovered in the neonate at birth or within placental tissue at delivery is also quantified. When maternal Fe status is compromised, a greater fraction of maternally absorbed Fe is transferred to the fetus and retained within the placenta relative to that recovered within maternal RBC's. Both maternal and neonatal hormones are associated with partitioning of absorbed Fe tracer. Neonatal hepcidin concentrations were strongly associated with Fe uptake by the fetus, indicating that the human fetus has some capacity to regulate placental Fe trafficking in a population at higher risk for maternal and neonatal anemia.

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CHAPTER 4

FETAL IRON UPTAKE FROM RECENT MATERNAL DIET AND RED BLOOD CELL CATABOLISM*

*Delaney KM, Cao C, Guillet R, Pressman EK, O'Brien KO. Fetal Iron Uptake from Recent Maternal Diet and Red Blood Cell Catabolism. AJCN *under review*

Abstract

Background: Iron across pregnancy can be obtained from the diet, body iron stores, or iron released from red blood cell (RBC) catabolism. Little is known about the relative use of these sources to support fetal iron acquisition.

Objective: To describe longitudinal change in iron absorption across gestation, RBC catabolism during pregnancy and partitioning of iron to the fetus.

Design: Fifteen pregnant women ingested an oral stable iron isotope (^{57}Fe) in the second trimester (T2) of pregnancy (week 14 -16) to label the RBC pool and a second oral stable isotope (^{58}Fe) in the third trimester (T3) (week 32 - 35). Absorption was measured at T2 and T3. Change in RBC ^{57}Fe and ^{58}Fe enrichment was monitored across pregnancy [18.8 – 26.6 weeks] to calculate RBC lifespan and quantify net iron loss from this pool. Iron transfer to the fetus was determined based on RBC ^{57}Fe and ^{58}Fe enrichment in umbilical cord blood at delivery.

Results: Iron absorption averaged 9% at T2 and increased significantly to 20% ($p = 0.006$) by T3. The net increase in iron absorption from T2 to T3 was strongly associated with net loss in maternal total body iron (TBI) from T2 to T3 ($p = 0.01$). Mean RBC lifespan during pregnancy was 94.9 days [95%CI: 43.5 – 207.1 days], and shorter RBC lifespan was associated with higher iron absorption in T2 ($p = 0.001$). An average of 87 ± 32 mg of iron was transferred to the fetus from maternal RBC catabolism and women with shorter RBC lifespans transferred more RBC-derived iron to their fetus.

Conclusion: Iron absorption doubled from T2 to T3 as maternal TBI declined. Women with low TBI had shorter RBC lifespans and transferred more RBC-derived

iron to their neonate. These findings suggest that the maternal RBC iron pool serves as a significant source of iron for the fetus particularly in women with depleted body iron stores.

Introduction

The developing fetus relies on placental transfer of iron from maternal sources such as: recently absorbed dietary iron, mobilization of hepatic or other body iron reserves or scavenging of iron released from catabolized red blood cells (RBC). While human and animal studies have assessed transfer of dietary iron to the fetus (1-6), little is known about the relative proportion of fetal iron that may be obtained from catabolism of maternal RBC's. In addition, little is known about factors that influence maternal RBC catabolism across pregnancy as only a few studies have monitored RBC lifespan across gestation (7-10). More work on understanding the dynamics of iron partitioning between the mother and her placental/fetal unit is needed to ensure that the mother has enough iron during pregnancy along with adequate neonatal iron endowment at birth is obtained.

Iron requirements increase by ~7-fold during pregnancy to over 7 mg/day in the third trimester (11). In recognition of these increased iron demands during pregnancy, the RDA for iron increases from 18 mg/d in non-pregnant women to 27 mg/d during pregnancy (12). This requirement was in part informed by early iron absorption estimations that were based on the acute recovery of an orally administered isotope in serum, an approach that does not reflect longer term utilization of iron for RBC production (13). Early studies were dependent on less precise mass spectrometric approaches. Furthermore, the data on maternally absorbed iron did not account for absorbed iron that was rapidly transferred to the fetus (14). Further understanding of maternal iron dynamics across gestation using current methodology is needed.

The majority of iron in the body is contained within the RBC pool. This pool recycles ~20 mg of iron daily as RBC's are catabolized. In non-pregnant women the iron that is released is either reutilized to support erythropoietic demands or stored. During pregnancy, some of this iron may be shunted to the fetus to support fetal iron demands. The RBC lifespan has been monitored in older studies using intravenous radioactive chromium (51Cr) or biotinylated labeled RBC's (15-21). These studies estimated the RBC lifespan to be ~120 ± ~30 days in adult men and women. Analogous data in pregnant women are lacking and studies assessing the RBC lifespan and fetal use of iron released from RBC catabolism are needed.

The developing fetus relies on maternal iron sources for adequate iron accretion in utero. It is likely that some of this iron is obtained from maternal RBC catabolism (22), but to date our understanding of iron recycling from RBC catabolism comes primarily from radioisotope studies in adult men (15-17). To fill these gaps in knowledge, the goals of this study were to 1) obtain longitudinal measures of maternal iron absorption from early to late gestation; 2) to evaluate RBC lifespan and determinants of RBC lifespan in pregnant women 3) to evaluate possible correlations between RBC lifespan and the amount of stable iron isotope recovered in the newborn at birth.

Methods

Participants

Twenty-three women were recruited from University of Rochester Medical Center Midwifery Group and from the Rochester Adolescent Maternity Program (RAMP) in Rochester, NY early in gestation (13-15 weeks of gestation). Women were

eligible to participate if they had an uncomplicated pregnancy, were carrying a singleton, were not previously treated for lead exposure, and did not have any malabsorption diseases, hemoglobinopathies or were taking a medication that might alter iron homeostasis. One participant withdrew before receiving the first iron tracer dose, three participants were ineligible due to subsequent health complications across gestation (gallbladder removal (n = 2), GDM (n = 1)), and dosing errors occurred in four women resulting in a final study population of fifteen women. The study was approved by Institutional Review Board of Cornell University and the University of Rochester Research Subjects Review Board and written consent was obtained from all participants (**Appendix Form 1)**.

Study Procedure and Isotope Dosing

This study was carried out from 2012 to 2015. All participants were asked to discontinue iron or prenatal supplement use two days prior to each stable isotope dosing study. **Figure 4.1** depicts the timeline for the study and the study measures collected at each visit (V1-V5). During the second trimester (T2) of pregnancy (V1: 14 - 16 weeks of gestation), women came to the RAMP clinic or Midwifery Group office after an overnight fast. Their height and weight were measured with a stadiometer and calibrated scale. A venous blood sample (5 mL) was collected to determine baseline iron status. To label the maternal RBC pool, each woman ingested a 20 mg dose of ^{57}Fe as ferrous sulfate in a small amount of flavored syrup (Humco; Austin, TX). Woman were then given a packaged lunch (canned vegetable soup and pretzels) and were instructed to wait at least 1.5 hours before consuming this meal. Women returned for 5 more visits over gestation to have blood samples taken: V2

(week 16 -18), V3 (week 24-26), V4 (week 33-35), V5 (week 35-37) and at delivery. The V2 sample was used to obtain the first measure of iron absorption based on 57Fe RBC enrichment 2-weeks post-dosing. Subsequent measures of ^{57}Fe RBC enrichment were obtained in V3, V4, V5 and at delivery to monitor the change in RBC enrichment from week 16–18 of pregnancy until delivery.

At V4, after the fasting blood sample was obtained, women ingested a second oral stable iron isotope (1.1 mg of ^{58}Fe and 18.9 mg iron as ferrous sulfate for a final total iron dose of 20 mg). They returned home with the same standardized lunch meal and were again instructed to wait at least 1.5 hours before consuming this meal. Iron absorption in the third trimester (T3) was measured 2-weeks post-dosing using the V5 sample based on ^{58}Fe RBC enrichment. At delivery, a cord blood sample (30 mL) was collected for analysis of neonatal iron status biomarkers and to determine the net transfer of ^{57}Fe and ^{58}Fe to the fetus based on neonatal RBC enrichment at birth.

Laboratory Analysis

Hemoglobin concentrations were measured using a HemoCue Analyzer (HemoCue; Brea, CA) at the University of Rochester. Serum was separated from whole blood and stored at -80°C until used. Serum ferritin (SF) and soluble transferrin receptor (sTfR) were measured using commercially available kits (Ramco Inc; Stafford, TX). Hepcidin and erythropoietin (EPO) were measured by commercially available ELISA kits (Bachem; Torrance, CA; R&D systems, Minneapolis, MN respectively). The limit of detection (LOD) for the hepcidin assay was 0.39 ng/mL and for statistical purposes a value of 0.195 ng/mL was assigned. Erythroferrone was measured with a commercially available ELISA (Intrinsic Lifesciences; La Jolla, CA),

and the LOD for this kit was 0.15 ng/mL. The kit provides quantitative measures below this level and measured values were used for analysis. Total body iron (TBI) was calculated from SF and sTfR as previously described (19, 20). All samples were run in duplicate.

Iron Isotope preparation

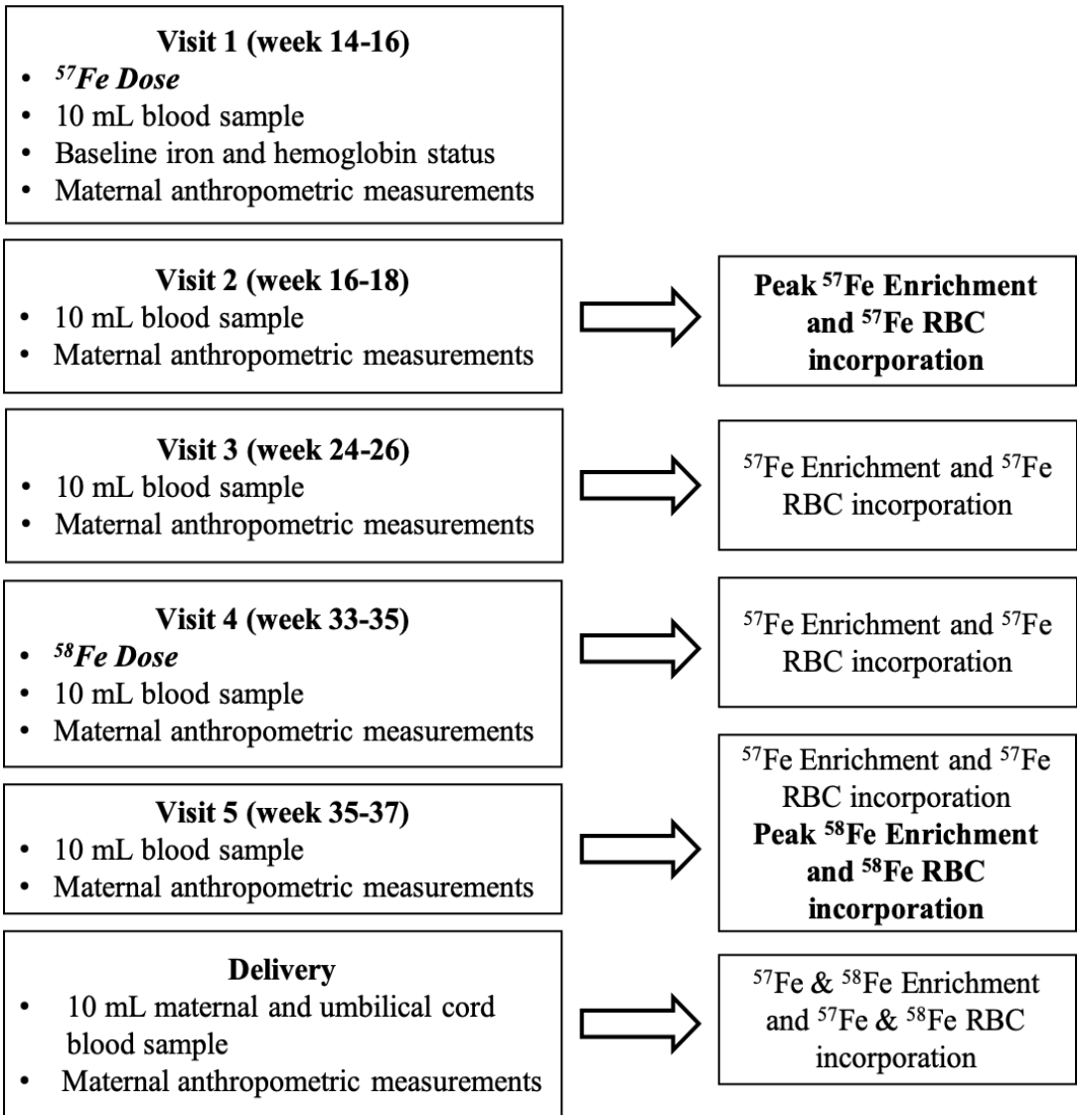
Iron isotopes (^{57}Fe at 94.69% enrichment and ^{58}Fe at 93.34% enrichment) were purchased in metal form (Trace Sciences International; Ontario, Canada) and were converted into a ferrous sulfate solution containing ascorbic acid at a 2:1 ratio as previously detailed (21). The isotopic composition of each tracer solution was validated using a Triton TI Magnetic Sector Thermal Ionization Mass Spectrometer (TIMS) (Thermo Fisher Scientific; Breman, Germany) and the total iron content of each tracer solution was measured using atomic absorption spectrophotometry Perkin-Elmer Analyst 800; PerkinElmer).

Isolation and Measurement of Iron Isotope

Maternal and umbilical cord blood (1 mL) was digested with 2 mL ultra-pure nitric acid (Ultrex, JT Baker). The digested residue was reconstituted in 2 mL ultra-pure hydrochloric acid (Ultrex, JT Baker) and Fe was extracted using anion exchange chromatography. The eluate was dried on a hot plate and reconstituted in 30 µL of 3% ultra-pure nitric acid (Ultrex, JT Baker). The extracted iron (8 µL) was loaded onto a degassed rhenium (H Cross, Moonachie, NJ) filament with 6 µL silica gel (Sigma-Aldrich, St. Louis, MO) and 2 µL phosphoric acid (Sigma-Aldrich, St. Louis, MO). Iron isotopic ratios ($^{57/56}$Fe, $^{58/56}$Fe, and $^{54/56}$Fe) were measured using Triton TI

Magnetic Sector Thermal Ionization Mass Spectrometer (TIMS) (Thermo Fisher Scientific; Breman, Germany).

Figure 4.1: Study Timeline for Dosing and Iron Analysis



The figure depicts each study visit and which week of gestation the visit will occur as well as measures collected at each visit. Iron enrichment and incorporation measured in blood collected at each visit is also described.

Calculation of Red Blood Cell Iron Enrichment

The blood samples taken 2 weeks post-dosing (V2) were used to measure maternal RBC enrichment. The 2-week post-dosing sample collection time is based on data obtained in men from early radiotracer studies that found the majority of ingested tracer was incorporated into RBC by two weeks post dosing (22). The delta percent (Δ %) excess of each iron isotope was calculated as the degree to which the iron isotopic ratios ($^{57/56}$Fe or $^{58/56}$Fe) differed from the baseline natural abundance ratios (0.02317 and 0.00307 respectively). Maternal circulating iron during pregnancy was calculated using measured maternal hemoglobin (Hb) concentration (g/dL) at each study visit, the estimated iron content of Hb (3.47 g/kg)(23), maternal weight (kg) at each study visit, and an assumed blood volume of 70 mL/kg (24). Using the Δ % excess and circulating iron concentration, the net quantity (mg) of tracer incorporated into the maternal RBC pool was calculated after assuming that 80% of absorbed iron was incorporated into erythrocytes (25).

Neonatal RBC iron incorporation was calculated using the same approach as detailed above except that the assumed blood volume was estimated as 80 mL/kg (1). We assumed that 80% of iron was incorporated into neonatal RBC based on data obtained from autopsy studies at birth (26-31). This amount is lower than the value of 90% that is often used in similar studies carried out in older infants (32) use of the 80% estimate is a more accurate estimate for a newborn given the dynamic changes that occur in iron redistribution over the early weeks of life.

We previously reported that absorption estimations are underestimated when only utilizing iron isotope incorporated into maternal RBC (10), as some tracer is

rapidly transferred to the fetus (2). Therefore, absorption was calculated as the net quantity (mg) of tracer incorporated into the maternal and neonatal RBC pool divided by the tracer dose consumed by the mother.

Approaches to Evaluate RBC Enrichment Change Across Pregnancy

The RBC ^{57}Fe enrichment at each timepoint was plotted over time. RBC half-life was calculated from using the equation; $T_{1/2} = \ln(2)/ \lambda$ where λ is the observed slope of change in RBC enrichment from V2 to V4 when observed change over time was linear. As $\ln(2)/ \lambda = \tau \ln(2)$, the average RBC lifespan (τ) can also be calculated. Random slope and intercept models were also used to assess longitudinal changes in RBC enrichment over time. Additionally, change in RBC enrichment across gestation was determined by two methods: 1.) the net change in RBC enrichment was calculated as difference between V2 to delivery and 2.) the slope of change was estimated for each woman individually using all timepoints. Finally, the area under the curve (AUC) for each participant was calculated using the trapezoidal method (33).

Approaches to Evaluate Net Transfer of Iron to the Neonate and Iron Partitioning

Net transfer of ^{57}Fe and ^{58}Fe to the fetus was examined using two approaches. Net transfer was calculated as the net mg of tracer recovered in neonatal RBC presented as a fraction of the amount of maternal tracer ingested and also as the net amount of tracer recovered in maternal RBC's 2-weeks post dosing. The net loss of ^{57}Fe (in mg) from the maternal RBC pool was also evaluated as a fraction of the total ^{57}Fe (in mg) recovered in the neonatal compartment.

Iron partitioning of ^{57}Fe between the maternal and neonatal compartment was assessed. Iron partitioning to the neonatal compartment was calculated as the net ^{57}Fe recovered (mg) in neonatal RBC as a fraction of the total ^{57}Fe recovered (mg) recovered in the maternal RBC pool two weeks post dosing. Similarly, iron partitioning to the neonatal compartment was calculated as net ^{58}Fe (mg) recovered in neonatal RBC pool as a fraction of total ^{58}Fe recovered in maternal and neonatal RBC pools.

Statistical Analysis

Participant characteristics and iron status indicators are presented as the mean ± SD, geometric mean [95%CI], or frequency and proportion. Possible changes in iron status indicators and participant characteristics between baseline and delivery were evaluated using t-test, Wilcoxon's rank sum test, or chi-square test as appropriate. Change in absorption was calculated as the difference in RBC iron enrichment between T2 and T3. Similarly, change in iron status indicators was calculated as the difference between each indicator from dosing week in T2 and T3. Linear regression models were used to assess correlation between variables and determinants and standardized beta coefficients (sβ) were reported. Statistical analyses were conducted with JMP Pro 14.

Results

Subject Characteristics and Iron Status

A total of 15 women completed the study. Maternal and neonatal characteristics are presented in **Table 4.1**. One participant delivered at week 35, before the second stable iron isotope could be administered and an additional woman

missed the second dosing visit. One of the participants in the study was an adolescent (17 years), and the rest were > 20 years of age. Based on self-report, none of the study volunteers were current cigarette smokers. Two neonates were born prematurely (13%) and one of the preterm neonates was classified as low birth weight (7%). At birth, 33% of newborns were anemic (cord Hb < 13 g/dL). Data on maternal and neonatal iron status are presented in **Table 4.2**. Maternal iron stores decreased significantly from baseline (~week 15) to delivery (SF $p = 0.03$; TBI $p = 0.01$), but prevalence of anemia did not significantly change across the study interval ($p = 0.23$).

Iron Absorption Across Gestation

Maternal iron absorption at T2 (15.0 ± 0.7 weeks) averaged 8.7% [95%CI: 5.0 – 15.4] and more than doubled across the ~19 week period that elapsed from T2 to T3 (at 34.0 ± 0.8 weeks) to 20.34 [95%CI: 13.82 – 26.86] ($p = 0.01$)(**Table 4.3**). The mean increase in absorption from T2 to T3 was 8.5 ± 11.6% and the mean change in TBI from T2 to T2 was -4 ± 4 mg/kg. For every 1 mg/kg decrease in total body iron from T2 to T3, iron absorption increased on average by 1.4% and change in absorption was significantly associated with change in maternal TBI status from T2 to T3. Change in total body iron from T2 to T3 explained 58% of variance in the change of iron absorption from T2 to T3. There was a trend for the change in iron absorption to be associated with the change in maternal hepcidin from T2 to T3 ($p = 0.06$). The observed increase in iron absorption was not significantly influenced by maternal age ($p = 0.5$), observed change in Hb from T2 to T3 ($p = 0.3$), maternal gestational weight gain from T2 to delivery ($p = 0.15$) or total gestational weight gain ($p = 0.09$). Total body iron at time of dosing explained 57% of variance in iron absorption in T2 ($p =$

0.003) and 34% of variance in iron absorption in T3 (p = 0.05), and TBI explained more variance in iron absorption than either SF (26%) or sTfR (18%) alone.

Table 4.1 Maternal and Neonatal Characteristics

Maternal Characteristics	Value
Age at entry (y)	27.31 ± 4.14
Race	
White (%)	67
Ethnicity	
Non-Hispanic (%)	86
Parity ≥ 1 (%)	56
Pre-pregnancy BMI	25.12 ± 7.99
Gestational weight gain (kg)	11.58 ± 11.02
Newborn Characteristics	
Gestational Age (wks)	39.23 ± 1.86
Birth weight (g)	3282.44 ± 451.37
LBW (%)[n]	6.7 [1/15]
Sex (% Female)	44

Data presented as mean ± SD or percentage.

Our data on maternal iron absorption was compared to previous published iron isotope data in pregnant women using orally administered iron tracers (**Appendix Table 2**). Results reported in the study by Hahn (34) were adjusted to assume 80% of absorbed iron was incorporated into RBC, as their original publication assumed 100% of orally absorbed isotope was incorporated into RBC's. The value of fold change increase in iron absorption we observed from T2 to T3 (2.2-fold increase) falls within the range that has been reported in the other studies, although the range reported in existing literature is wide. Fold increases in absorption ranged from 1-10 fold when evaluated from week 10-14 of gestation to >30 weeks of gestation.

Change in Maternal Red Blood Enrichment Across Gestation

To obtain an estimate of iron released from RBC catabolism across gestation, changes in RBC enrichment of ^{57}Fe were monitored over a 150-day period post-dosing (**Table 4.3, Figure 4.2**). Change in RBC ^{57}Fe enrichment was non-linear ($p = 0.02$), as there was a linear decrease in RBC enrichment from V2 to V4 followed by a slight increase in RBC enrichment at V5 and delivery. Because tracer recovery is based on estimates of maternal RBC mass, this non-linear change in RBC enrichment may be driven by the observed change in Hb across gestation (**Table 4.3**). Our observed changes in RBC enrichment and Hb across gestation mirror reported data on changes in hematocrit across gestation (**Figure 4.2**) (39). When assessing the decrease in RBC enrichment from V2 – V4, RBC enrichment decreased on average 0.03% per day over the 118 days ($p = 0.08$). As RBC enrichment was strongly inversely associated with TBI, controlling for TBI across gestation strengthened the relationship between RBC enrichment decrease per day (0.04% per day, $p = 0.04$). The calculated RBC lifespan, using only the linear portion of the half-life curve (V2 to V4), averaged 94.9 days [95%CI: 43.5 – 207.1 days] while the half-life from V2 to V4 averaged 66 days [95% CI: 30 – 144]. Women with shorter RBC lifespan absorbed significantly more iron in T2 ($\beta = -0.84$ $p = 0.001$).

Table 4.2: Longitudinal Changes in Maternal Iron Status and Neonatal Iron Status

	Trimester 2 (57 dose)	Trimester 3 (58 dose)	Delivery	Neonate
Gestational Age	14.97 ± 0.67	34.0 ± 0.74	39.41 ± 1.78	39.41 ± 1.78
N	15	13	15	15
Weight (kg)	68.92 ± 21.20[a]	77.68 ± 18.78[b]	79.85 ± 20.73[c]	3.36 ± 0.33
Hemoglobin (g/dL)	12.17 ± 1.88	10.98 ± 1.30	11.64 ± 1.24	13.72 ± 2.31
Anemia (%)	7	54	23	33
Serum Ferritin (ug/L)	50.12 [30.56 – 82.19][a]	22.70 [12.05 – 42.76][b]	33.75 [17.64 – 64.58][c]	205.82 [141.53 – 299.31]
Serum Ferritin < 12 ug/L (%)	7	25	8	-
Transferrin Receptor (mg/L)	3.64 [2.64 – 5.02][a]	4.91 [3.63 – 6.63][b]	5.31 [3.83 – 7.37][c]	6.20 ± 1.82
Total body iron (mg/kg)	8.04 ± 3.39[a]	4.02 ± 3.54[ab]	5.18 ± 4.13[b]	11.29 ± 2.95
Erythroferrone (ng/mL)	0.74 [0.37 – 1.46]	0.79 [0.38 – 1.65]	0.68 [0.36 – 1.27]	1.62 [0.80 – 3.31]
Erythropoietin (mIU/mL)	18.51 [12.38 – 27.68][a]	30.35 [17.61 – 52.32][b]	29.46 [16.33 – 53.17][a]	27.35 [16.64 – 44.95]
Hepcidin	10.78 ± 7.50	5.44 ± 5.13	-	35.96 ± 18.17
Undetectable (%)	7	0		0

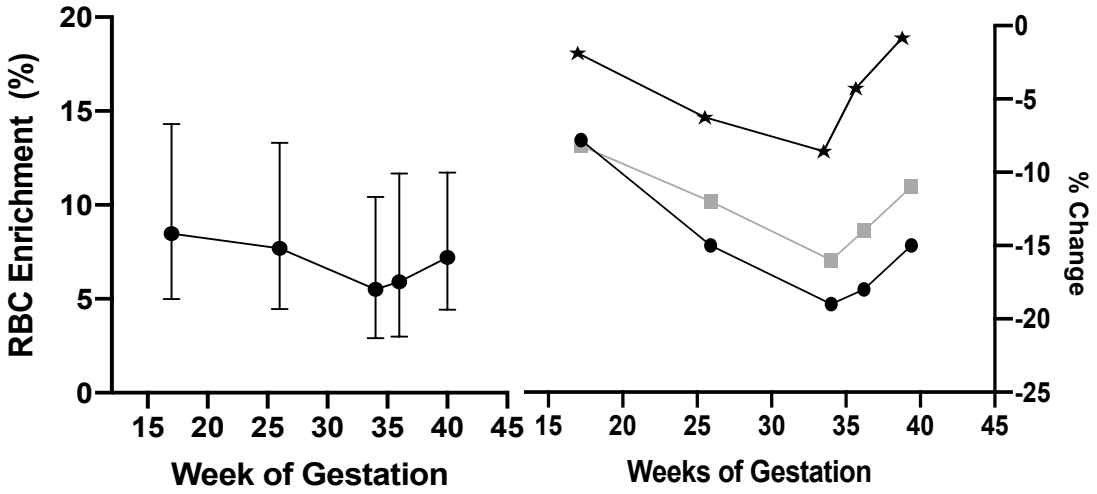
Data presented as mean ± SD for normally distributed data or geometric mean [95%CI] for non-normally distributed data. Values with different superscripts indicate trimester 2, trimester 3 and/or delivery significantly differed, $p < 0.05$.

Table 4.3 Iron Enrichment, Weight and Hb Measures Across Gestation

	Visit 1 (wk 14-16)	Visit 2 (wk 16-18)	Visit 3 (wk 24-26)	Visit 4 (wk 33-35)	Visit 5 (wk 35-37)	Delivery (wk 35-41)	Neonate
N	15	15	13	13	13	15	15
Weight (kg)	68.9 ± 21.2	70.1 ± 20.6	70.2 ± 17.1	77.7 ± 18.8	77.9 ± 19.1	79.8 ± 20.7	3.31 ± 0.5
Hb (g/dL)	12.2 ± 1.9	11.9 ± 1.5	11.2 ± 1.0	11.0 ± 1.3	11.5 ± 1.3	11.6 ± 1.2	13.7 ± 2.3
Fe^{57} Δ% excess	-	3.18 ± 2.1	2.92 ± 2.1	2.06 ± 1.3	2.08 ± 1.3	2.07 ± 1.2	2.21 ± 1.37
Fe^{57}mg	-	1.04 [0.6 - 1.8]	0.94 [0.5 – 1.6]	0.67 [0.4 – 1.3]	0.73 [0.4 – 1.4]	0.81 [0.5 – 1.3]	0.06 ± 0.04
Fe^{57} RBC enrichment (%)	-	8.48 [5.0 - 14.4]	7.69 [4.5 - 13.3]	5.50 [2.9 – 10.4]	5.91 [3.0-11.7]	7.21 [4.4 – 11.7]	0.47 ± 0.3
Fe^{58} Δ% excess	-	-	-	-	2.44 [1.6 – 3.7]	2.81 [2.1 – 3.9]	3.58 ± 1.9
Fe^{58} mg	-	-	-	-	0.15 [0.1 - 0.2]	0.18 [0.1 – 0.2]	0.01 ± 0.01
Fe^{58} RBC enrichment (%)	-	-	-	-	19.57 ± 8.7	21.86 ± 10.5	

Data presented as mean ± SD for normally distributed data or geometric mean [95%CI] for non-normally distributed data.

Figure 4.2 Change in RBC enrichment across gestation



The left panel depicts observed changes in RBC 57Fe enrichment (%) over gestation in 15 pregnant women. Data are presented as geometric mean and 95% CI. The right panel presents the observed change in Hb (black Stars, n is the same as left panel 13-15) in these women across gestation from visit 1 (week 14-16). The observed changes in Hct in non-iron supplemented women (black circles, n = 39) and Hct in iron supplemented women (gray squares, n = 39) from pre-pregnancy data adapted from Vricella 2017 (39).

Net mass of ^{57}Fe present in the RBC pool was also calculated at each timepoint. An average of 11% [95% CI: 2.6 – 20.9] of total ^{57}Fe mass incorporated into RBC was lost over the ~150-day period from V2 to delivery (0.12% per day p = 0.03). Assuming the tracer was equilibrated in the RBC pool, this magnitude of loss would reflect a net loss of 295 mg [95% CI: 145 – 602] from the RBC pool over the ~150 days. The AUC for RBC enrichment across the 150-day period was also calculated. The AUC was greater in women who absorbed more ^{57}Fe in T2 ($s\beta$ = 0.57, p = 0.04), and these women also experienced a significantly greater decrease in RBC enrichment over time (p= 0.04).

Net Transfer of Iron Released From RBC Catabolism to the Neonatal Compartment

The net mg quantity of ^{57}Fe recovered in neonatal RBC was 60 ± 40 μg, which translates to 0.5% [95% CI: 0.28, 0.66] of the 20 mg ^{57}Fe dose consumed by the mother at week 13 of gestation. With the average absorption of 8% in these women, the 60 μg recovered in neonatal RBC translates to 5.9% [95% CI: 4.5, 7.2] of the amount of ^{57}Fe absorbed by the mother. The amount of ^{57}Fe recovered in the neonatal RBC pool was not a function of the time that elapsed between dosing and delivery ($p = 0.94$), which averaged 170 days in this cohort. When assessing ^{57}Fe released from maternal RBC, a subset of neonates obtained more ^{57}Fe than was released (n = 6). These neonates were born to mothers who absorbed less iron ($p = 0.1$), longer RBC lifespan (63.8 days vs. 164.5 days; $p = 0.3$) and thus less iron released from RBC catabolism (0.04 vs 0.42 mg, $p = 0.001$). In the neonates who obtained less ^{57}Fe than was released (n = 7), the net ^{57}Fe in the neonatal compartment corresponded to 20.5% [Range: 14.4 – 54.7%] of the ^{57}Fe released from maternal RBC turnover. Greater % ^{57}Fe released from maternal RBC that was recovered in the neonatal compartment was associated with a greater decrease in maternal TBI ($p = 0.1$).

Net ^{57}Fe recovered in neonatal RBC (mg) was strongly positively associated with the amount of iron absorbed by the mother at T2 ($R^2 = 0.78$ $p < 0.001$) and with maternal ^{57}Fe AUC ($R^2 = 0.73$, $p = 0.0002$). Women who exhibited a greater decrease in RBC enrichment over the 150 days between dosing and delivery, (calculated either as a slope from V2 to delivery or as change in ^{57}Fe (mg) over this interval), transferred more ^{57}Fe to their neonate (slope: $s\beta = -0.69$, $p = 0.02$; change $s\beta = -0.76$, $p = 0.003$).

Consequentially, women who had shorter RBC half-lives transferred more net 57Fe (mg) to the neonate (sβ = -0.63, p = 0.04).

Net Transfer of Dietary Iron to the Neonatal Compartment in the Third Trimester

The net ^{58}Fe recovered in the neonatal RBC was 10 ± 10 μg, which reflects 1.3% [95% CI: 0.9, 1.7] of the ^{58}Fe dose consumed by the mother at ~week 34 of gestation. With the average absorption at this time being nearly 20%, the 10 μg recovered in neonatal RBC translates to 9.0% [95%CI: 5.8 – 13.9%] of the amount of ^{58}Fe absorbed by the mother. The amount of ^{58}Fe recovered in the neonatal RBC pool was not a function of the time elapsed between dosing and delivery (p = 0.5) which averaged 42 days. Net ^{58}Fe recovered in the neonatal RBC was not as strongly associated with absorbed iron in T3 (p = 0.06) as seen with ^{57}Fe. Net ^{58}Fe recovered in neonatal RBC was inversely associated with maternal delivery SF (sβ = -0.70, p = 0.03), and was more strongly associated with SF than TBI (sβ = -0.61, p = 0.06).

Iron Partitioning Between Maternal and Neonatal Compartments

The total amount of each of the ^{57}Fe and ^{58}Fe tracer recovered in the maternal and neonatal compartment was summed, and the relative fraction of the total iron partitioned to the maternal or neonatal compartment was calculated. Of the total ^{57}Fe recovered in the mother 2 weeks post-dosing and in the newborn, 4.7% [95%CI: 3.6% – 5.8%] was partitioned to the neonatal compartment. The fraction of total ^{58}Fe recovered that was partitioned to the neonatal compartment was higher, although this did not reach statistical significance (6.5% [95% CI: 4.4% – 9.8%], p = 0.12). Neonates with a larger fraction of total ^{57}Fe recovered partitioned to their RBC also

had a higher fraction of total ^{58}Fe recovered partitioned to their compartment (R^2 = 0.75, p = 0.006). Partitioning of each isotope to the neonatal compartment was positively associated with neonatal ERFE concentrations at birth (57Fe: sβ = 0.61, p = 0.03; 58Fe: sβ = 0.56 p = 0.06). Women with greater change in TBI across gestation partitioned a greater fraction of orally ingested iron to their neonatal compartment (^{57}Fe p = 0.03; ^{58}Fe p = 0.08).

Discussion

To our knowledge this is the first longitudinal isotope study that sought to quantify the kinetics of RBC iron turnover during pregnancy. Furthermore, these longitudinal data, and use of two stable iron isotopes, allowed for the elucidation of relationships between a women's iron absorption capacity from T2 to T3 as a function of decreasing iron stores. We report that mean RBC lifespan averaged 95 days during pregnancy, and women with a shorter RBC lifespan absorbed more iron in early gestation and transferred a larger net fraction of absorbed iron to their neonate.

In the second trimester of pregnancy, iron absorption averaged ~ 9% and doubled to more than 20% in the third trimester. The increase in absorption is consistent with early data, and our 2.2 fold increase in absorption fell within the range of fold increase previously observed [range 1.3 – 10 fold increase] (**Appendix Table 2**). In the current study, the percent iron absorption at T3 (20%) was lower than previous stable iron isotope studies (ranging 36% – 66%) (14, 28, 29, 33). This may be due to the total load of iron given (20 mg), which was higher than that used in previous studies (5 – 10 mg), and iron absorption is known to decrease as the load of iron ingested increases (38). Additionally, the women in this study appear to be more

iron replete (higher SF or TBI) than those in previous studies. Maternal iron absorption is inversely associated with maternal iron status (13, 14, 29, 40). This finding is also evident in the current study where women had greater increases in iron absorption from T2 to T3 when they exhibited a greater loss in total body iron from T2 – T3. While we did observe inverse associations between SF and iron absorption, maternal TBI was more strongly predictive of maternal iron absorption than was hepcidin, a finding that is consistent with other data in non-pregnant adults (41).

RBCs are anucleate and have a finite life span. While there was a wide range in variability in RBC lifespan [95% CI: 43.5 – 207.1 days days], RBC lifespan during pregnancy averaged 95 days. The range of RBC lifespan in our study is much greater than the range of 90 - 130 days reported in non-pregnant women and adult men (19, 42-44). While we did not see a linear decrease in RBC enrichment over pregnancy, we found that our change in RBC enrichment mirrored observed changes in maternal Hb and was also consistent with expected shifts in plasma volume reported from normative data (39) (**Figure 4.2**). There are few published data on RBC lifespan during pregnancy that can be used for comparison purposes. Only one study to our knowledge has estimated RBC lifespan at 120 days in a small group of pregnant women (n=6) using RBC's that were removed, labeled ex vivo with ^{51}Cr, and then reinfused (7). Studies in pregnant rats that also labeled RBC's ex vivo with ^{51}Cr found that RBC lifespan was shortened by 9% during pregnancy compared to values obtained in non-pregnant rats (36.9 days to 33.6 days, $p = 0.001$) (10). However, the relevance of data from rats may be limited given the many documented differences in iron metabolism between rats and humans (45, 46). Other data have estimated RBC

lifespan in adult men and non-pregnant women using ex vivo labeling of RBC with 51Cr or biotin, and with these approaches RBC lifespan in non-pregnant women and adult men ranged from 110-130 days (15-21). Although methodological approaches differ, our findings suggest RBC lifespan may be ~20% shorter than prior values observed in non-pregnant women. Further studies are needed to evaluate this measure in larger sample sizes that include both pregnant and non-pregnant women.

The developing fetus relies on maternal iron stores and efficient placental transport of iron across gestation. The current study found that iron recycled from RBC catabolism provides a significant source of iron for the fetus, and reliance on this pool of iron is greatest in women with low body iron. The majority of RBC catabolism occurs extravascularly, releasing ~20 mg of iron back into circulation as di-ferric transferrin (47) and the placenta is known to upregulate transferrin receptor in relation to maternal iron status so it may better capture diferric-transferrin (48). However, 10-20% of RBC catabolism occurs intravascularly, releasing free Hb and heme, that bind to their respective carriers (17, 47). The placenta also expresses a number of heme transport proteins (49-56). Whether the iron released from RBC catabolism is transferred to the neonatal compartment as diferric- transferrin or within a heme containing protein is unknown, but RBC catabolism appears to be a significant source of iron for the fetus and this pool is utilized to a far greater extent in women with depleted iron reserves.

Although this study assessed RBC dynamics in pregnancy and iron transfer derived from RBC catabolism during pregnancy, there are limitations. The current sample size is limited, and we do not have data on iron absorption and change in RBC

enrichment across a similar interval of time in non-pregnant women to serve as a control. We dosed women with a large load of iron (20 mg) which could lead to an underestimation of true iron absorption. We also assumed a constant blood volume as a function of maternal weight across gestation, although we know that plasma and blood volume increase from 12 to 28 weeks of gestation and then plateaus (39), with plasma volume nearly doubling in T3 compared to non-pregnant values (57). Furthermore, these methods have not been optimized during pregnancy, which likely resulted in large variability in RBC lifespan during pregnancy. We were also unable to distinguish the relative amount of neonatal ^{57}Fe that was derived from rapid transfer of ingested iron to the fetus versus that obtained from RBC catabolism. Further studies are needed with larger sample sizes to confirm these results.

In conclusion, the current longitudinal study has found that iron absorption increases across gestation as a function of decreasing maternal total body iron. We also report for the first time that the lifespan of RBC during pregnancy averages 95 days and is shortened in women with diminished iron stores. The women with shorter RBC lifespan transfer a greater net amount of iron to their neonates, suggesting in times of increased iron demand RBC iron becomes a significant source for fetal iron.

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CHAPTER 5

BIOLIGICAL IMMATURITY IMPACTS PLACENTAL ERYTHROFERRONE AND ERYTHROPOIETIN EXPRESSION*

*Delaney KM, Castillo L, Ronnie Guillet[2], Eva K. Pressman[3], Thomas Ganz[4], Elizabeta Nemeth[5], Kimberly O. O'Brien[1] Biological Immaturity Impacts Placental Erythroferrone and Erythropoietin Expression

Abstract

Background: Iron homeostasis is controlled by the hormones erythroferrone (ERFE), erythropoietin (EPO) and hepcidin, all of which are expressed in the human placenta. At this time, determinants of the placental expression of these hormones and if they are associated with maternal or neonatal iron status is unknown.

Objective: To characterize the expression of placental ERFE, hepcidin and EPO mRNA in placentae obtained from women at increased risk of iron deficiency.

Methods: Placentae were collected from 114 neonates born to adolescents carrying singletons and 110 neonates born to 54 women carrying multiples. Placental EPO and ERFE mRNA expression in placental tissue was measured by qPCR and compared to existing data on iron status and iron regulatory hormones in these cohorts.

Results: Placental ERFE and EPO mRNA was detectable in all placentae delivered between 25 – 42 weeks of gestation, but relationships between placental EPO and ERFE differed by cohort. In the multiples cohort, placental EPO and ERFE expression were positively correlated ($p = 0.004$), but there was only a trend for a positive association between placental EPO and ERFE ($p = 0.08$) in the adolescent cohort. In this cohort no consistent relationships were observed between EPO and ERFE expression and maternal or neonatal iron status biomarkers.

Conclusion: The human placenta expresses ERFE and EPO as early as 25 weeks of gestation. Placental expression of ERFE and EPO were not significantly related to maternal or neonatal iron status or iron regulatory hormones. Further research is needed to identify the role of these hormones in the human placenta.

Introduction

The placenta is a dynamic, fetally-derived organ that serves as the sole interface between the mother and fetus. The placenta must respond to competing demands as it must support its own high metabolic requirements, while simultaneously transferring nutrients to the fetus in response to maternal and fetal signals (1). Efficient placental iron transport is essential to support fetal growth and prevent adverse neonatal outcomes (2, 3). Three regulatory hormones, hepcidin, erythropoietin (EPO) and erythroferrone (ERFE), are involved in iron homeostasis and work in concert to regulate iron absorption and utilization in the mother and her developing fetus. The placenta also expresses all three hormones, although the functions of these hormones in this organ are not fully understood.

Hepcidin is the master regulator of iron status, and this hormone functions to decrease circulating iron by binding and inhibiting ferroportin mediated export of iron from cells (4, 5). Our previous data in placentae collected from the adolescent cohort found that placental hepcidin mRNA expression was unrelated to expression of placental iron transporters or maternal and neonatal iron status (6). Hepcidin also functions anti-microbial peptide,(7) and placental hepcidin expression is increased in women with malarial infection, suggesting that this hormone may act as a placental antimicrobial peptide (8).

Erythropoietin is produced in response to hypoxia to increase circulating iron concentrations (9, 10). Placental EPO mRNA and protein have been detected in placental tissue from animals and humans (11-14), although data on the relationship of placental EPO with placental iron transport proteins or maternal and neonatal iron

status are limited. A study in sheep found placental EPO expression increased significantly under hypoxic conditions (14), and one study in human twin pregnancies (n = 26) found placental EPO expression was induced by neonatal hypoxia (confirmed by umbilical artery doppler) and severe growth restriction (13).

The third iron-related regulatory hormone, ERFE, was recently discovered but expression of this hormone in placental tissue has yet to be evaluated in animal or human placentae. The objective of this paper was to measure placental ERFE and EPO mRNA expression in relation to maternal, fetal and placental iron status and placental hepcidin mRNA expression in placental tissue obtained from newborns delivered to women and teens at higher risk of iron deficiency and anemia.

Methods

Participants:

Placental tissue was obtained from two study cohorts, women (n = 54) carrying multiple fetuses (placentae from twins n = 73, placentae from triplets n = 37) (15, 16), and pregnant adolescents (age < 19) years carrying singletons (n = 114) (17, 18) to assess relationships between placental expression of iron transport genes in relation to nutritional status and biochemical markers in the mother and neonate. Pregnant adolescents were recruited from the Rochester Adolescent Maternity Program (RAMP) in Rochester, NY between 2006 – 2012 and women carrying multiples were recruited from Strong Memorial Hospital and Highland Hospital in Rochester, NY from 2011 - 2014. Descriptive data on iron status from the pregnant teens/women and women carrying multiples have previously been published (16-24). Women carrying multiples were eligible if they were healthy and ≥19 years of age and pregnant women

and teens were eligible if they were healthy and carrying a single fetus. In all studies individuals 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 teen assent were obtained from adolescents ≤ 14 y of age. All studies were approved by the Institutional Review Boards at Cornell University and the University of Rochester.

Demographic information was self-reported upon entry to the study. Gestational age (GA) was determined based on self-reported menstrual history and sonogram data, or by date of in-vitro fertilization (IVF) when applicable in the multiples cohort. If self-reported menstrual history and sonogram data differed by more than ten days, ultrasound estimates were used to determine gestational age. Maternal anthropometric information was recorded at each study visit and at birth, infant weight and length were recorded and cord blood and placental tissue was obtained. Gestational weight gain (GWG) was calculated as the difference between final weight at delivery and self-reported height and pre-pregnancy weight.

Placental collection:

At delivery, placentae were collected and placental weight and dimensions (length, width, thickness, and volume) were recorded by study staff (adolescent participants) or by the University of Rochester Pathology Department (multiples cohort). The majority (70%) of the multiples placentae were fused, so placental weight in fused placentae were obtained by dividing the entire placental mass by the number of placentae. Maternal and fetal membranes and superficial tissue on the maternal side

were removed and placental samples were excised from multiple cotyledons of each placenta, which were pooled into a representative sample. The mixture was either placed into RNAlater (Ambion, Austin, TX, USA) and kept at -80□ C until analysis. Total placental iron content (p[Fe]) of each placenta was analyzed by inductively coupled plasma-mass spectrometry (25). Data on placental transferrin receptor expression(26), feline leukemia virus subgroup C receptor (27), LDL receptor-related protein 1 (28), mineral content (25), and vitamin D hydroxylase enzymes and vitamin D receptor (29, 30) have been previously reported.

Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

For all placentae, total RNA was extracted using the RNeasy Microarray Tissue Mini Kit (Qiagen, Valencia, CA, USA) and RNA purity was checked by the ratio of absorbance at 260 and 280 nm on a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A total of 1 μg RNA was reverse-transcribed into cDNA with the transcriptor cDNA synthesis kit (Roche Applied Sciences, Indianapolis, IN, USA). All qRT-PCR reactions used a total volume of 10 μL per reaction, which contained 2 μL of the cDNA template, 5 μL of SYBR Green I Master reaction mix, and 0.7 μM of primers and samples were run in triplicate in 384-well plates on a LightCycler 480 instrument (Roche Applied Sciences, Indianapolis, IN, USA). The cycling conditions for ERFE included an initial denaturation step at 95°C for 5 min, followed by 45 cycles at 95°C for 10 sec, annealing at 63°C for 20 sec, and extension at 72°C for 15 sec. The cycling conditions for EPO involved an initial denaturation step at 95°C for 5 min, followed by 45 cycles at 95°C for 10 sec,

annealing at 62°C for 15 sec, and extension at 72°C for 20 sec. Specificity of amplifications was verified by melt curve analysis and only a single peak was observed in all samples. Relative ERFE and EPO mRNA expression was normalized to β-actin and compared to that obtained from a control placenta sample using the 2-ΔΔCt method. This method was calculated using the following equations: ΔCt = Ct(EPO, ERFE or hepcidin) − Ct(β-actin); ΔΔCt = ΔCt(sample) − ΔCt(control placenta); and Fold Change = 2−ΔΔCt. The primer sequences for ERFE, hepcidin and β-actin have been previously published (6, 28, 31). The primer sequences for EPO were as follows: forward 5′-AGAATATCACGACGGGCTGT-3′ and reverse 5′-AGGCCCTGCCAGACTTCTAC-3′.

Serum collection and biochemical analysis:

Nonfasted maternal blood (15 mL) was collected from women at mid-gestation (~26 weeks) and when admitted to the hospital for delivery (~39 weeks) and umbilical cord blood (~15 mL) was collected at delivery. Whole blood was sent to the University of Rochester core laboratory for assessment of hemoglobin (Hb) concentrations using a Cell-Dyn 4000 hematology analyzer (Abbott diagnostics, Santa Clara, CA). The remaining blood samples were centrifuged, separated and serum was stored at -80□ C until analysis. Anemia across pregnancy was defined for Caucasians as hemoglobin concentration < 11.0 g/dL in the first and third trimesters and < 10.5 g/dL in the second trimester and for African Americans < 10.2 g/dL in the first and third trimesters and < 9.7 g/dL in the second trimester (32).

Serum ERFE was measured using a validated ELISA with a stated lower limit of detection (LOD) of 1.5 ng/mL (Intrinsic Lifesciences, La Jolla, CA). The ERFE

assay provides quantitative measures of ERFE down to 0.001 ng/mL so absolute values of this hormone were utilized for statistical analyses. Serum EPO was measured by immunoassay (Siemens Immulite 2000, Erlangen, Germany). Serum ferritin (SF), soluble transferrin receptor (sTfR) and hepcidin were measured by ELISA as previously described (16, 17) and iron deficiency was defined using the proposed cutoffs of either serum SF < 12 μg/L(33) or sTfR > 8.5 mg/L (34). Total body iron was calculated using SF and sTfR as previously described (35). Serum iron was measured by atomic absorption spectrophotometry (Perkin Elmer AAnalyst 800, Waltham, MA). Folate and vitamin B12 were measured by an Immulite 2000 immunoassay system (Siemens Healthcare, Erlangen, Germany). Folate insufficiency was defined as folate concentrations < 6.8 nmol/L and vitamin B12 insufficiency was defined as vitamin B12 concentration < 148 pmol/L (36, 37). Hepcidin, C-reactive protein (CRP) and interleukin-6 (IL-6) were measured using different assays between cohorts. For the adolescent cohort, hepcidin was measured with an ELISA and IL-6 and CRP were measured using a commercial immunoassay as previously described (17). The LOD for hepcidin was 5 □g/L, and values below were assigned a value of 2.5 □g/L for analysis purpose. For the multiples cohort, hepcidin, CRP and IL-6 were measured by ELISA as previously described (16). The hepcidin assay had a LOD of 0.39 ng/mL, and a value of 0.195 was assigned for analysis purpose.

Statistical analysis:

Placental characteristics are presented as median [95% CI] or percent for continuous and categorical outcomes respectively. The Shapiro-Wilk test was used to assess normality of data, and non-normal 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 the Wilcoxon's rank-sum test was used to test statistical differences between non-parametric 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 relationships between placental hormone expression and iron status indicators. Multiple regression was used to determine predictors of placental hormone expression beginning with variables identified by bivariate correlation with p values < 0.2 simultaneously tested and eliminated by backward selection until only statistically significant predictors remained. Placental efficiency was assessed as placental weight (g) / birth weight (g)(38, 39). The intraclass correlation coefficient (ICC) for ERFE was calculated as the between-mother variance divided by the sum of the within-mother and between-mother variances as previously published (22). Statistical analyses were performed using JMP 14.0 (SAS Institute Inc).

Results

Placental Characteristics

Expression of placental ERFE and EPO mRNA was evaluated in 114 placentas from the adolescent cohort and 110 placentae from the multiples cohort. Placental characteristics of these placentae, along with maternal and neonatal characteristics, are presented in **Table 5.1**. The majority (65%) of placentae from the multiples cohort were delivered pre-term. Additionally, each individual placenta from the multiples cohort weighed significantly less than observed in placentae from the adolescent cohort, however total placental mass per mother in the multiples cohort was significantly greater than the adolescent cohort ($p = 0.01$). There was a significantly higher percentage of placentae delivered by women with parity > 1 in the multiples cohort ($p < 0.001$). Placental efficiency was significantly greater in the multiples cohort ($p < 0.001$). As placental efficiency increased, neonatal erythrocyte iron content (mg/kg) increased ($p = 0.002$). This relationship remained significant after controlling for gestational age or maternal Hb status. Additionally, iron content (p[Fe]) (µg/g wet weight) was measured in a subset of placentae (Multiples: n = 65, Adolescent: n = 77), and p[Fe] was not significantly different between cohorts ($p = 0.31$).

Table 5.1: Characteristics of Mothers, Their Newborns and Placenta

	Multiples Cohort	Singletons Cohort	p-value
Placental characteristics	**(n = 110)**	**(n = 114)**	
Gestational age (wks)	35. [34.5, 35.6]	40.2 [39.7, 40.2]	**< 0.001**
Placenta weight per fetus (g)	297 [278.2, 316.0]	586 [577.9, 622.6]	**< 0.001**
Sum placental weight (g)	667 [606.9, 738.7]	586 [577.9, 622.6]	**0.01**
Maternal characteristics	**(n = 54)**	**(n = 114)**	
Age	30 [29.4, 32.1]	17.4 [17.2, 18.1]	**< 0.001**
Race (% Black)	22	64	**< 0.001**
Ethnicity (% Hispanic)	7	26	**0.002**
ppBMI (kg/m^2)	26.1 [26.4, 31.0]	23.0 [23.7, 25.8]	**0.001**
GWG (kg)	18.6 [17.1, 22.2]	18.2 [16.7, 19.4]	0.22
Parity (< 1 %)	37	79	**< 0.001**
Neonatal characteristics	**(n = 110)**	**(n = 114)**	
Birth weight (g)	2.2 [2.1, 2.3]	3.3 [3.2, 3.4]	**< 0.001**
Birth length (cm)	47.0 [44.7, 46.4]	51.5 [50.9, 51.8]	**< 0.001**
Sex (% male)	43	51	0.19
c-section (%)	73	11	**< 0.001**
Placenta/Fetus weight	0.13 [0.13 – 0.15]	0.18 [0.18 – 0.19]	**< 0.001**

Data presented as median [95% CI].

Placental Erythroferrone and Erythropoietin mRNA Expression

Placental ERFE and EPO mRNA was detectable in all placental samples analyzed from deliveries that occurred across the interval between 25 to 42 weeks of gestation. Placental ERFE mRNA expression was significantly higher in the multiples cohort ($p = 0.001$)(**Figure 5.1**), even after controlling for gestational age at delivery ($p = 0.004$), placental weight ($p = 0.01$), or maternal Hb ($p = 0.002$). Placental EPO mRNA expression was not significantly different between cohorts ($p = 0.17$). Placental EPO expression was significantly higher than placental ERFE expression in both cohorts (both $p < 0.001$) (**Figure 5.1**) and in the multiples cohort there was a significant positive relationship between placental EPO and ERFE ($\beta = 0.16$, $p = 0.004$, $n = 110$) which was not evident in the adolescent cohort ($\beta = -0.07$, $p = 0.08$, n

= 114). In both cohorts, placental ERFE and EPO did not significantly differ as a function of placental weight per fetus, neonatal birth weight, placental efficiency, or neonatal sex.

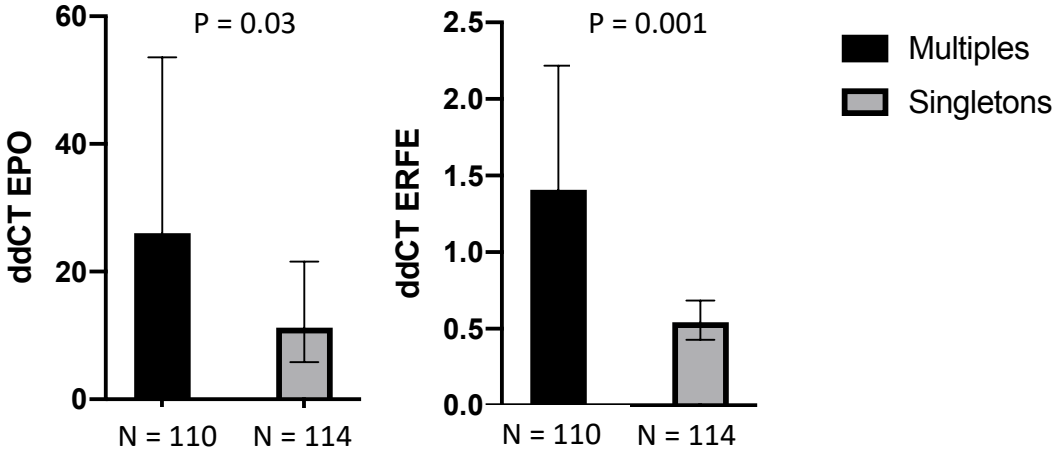

Figure 5.1: Expression of placental hormones (ddCT) in placental tissue obtained from neonates born to women carrying multiples or women and teens carrying singletons. Data presented as geometric mean [95% CI].

In the adolescent cohort, placental ERFE mRNA expression increased as gestation progressed from 34 – 42 weeks ($\beta = 0.11$, $p = 0.002$), but this relationship was not evident in the multiples cohort across the range of delivery from 25 – 38 weeks ($p = 0.9$) nor when restricting the data to the gestational window of 34 – 39 weeks to more closely match the adolescent cohort ($p = 0.5$). Placental ERFE did not significantly differ between Black and White mothers (ERFE: Multiples: $p = 0.2$, Adolescents: $p = 0.1$). Placental EPO expression did not significantly change as a function of gestational age in either cohort (Multiples: $p = 0.8$, Adolescents $p = 0.1$) however, placental EPO expression did significantly differ by race in both cohorts (Multiples: $p = 0.02$, Adolescents: $p = 0.03$). This relationship appeared to be driven by differences in maternal Hb (both $p = 0.1$).

Placental Hormone Relationships in Women Carrying Multiples

Placental EPO and ERFE expression in the multiples cohort were significantly inversely associated with maternal iron and hematological status. Placental EPO expression was significantly higher in gravida with evidence of limited iron status (ie. when maternal Hb was lower ($\beta = -0.20$, $p = 0.06$, $n = 110$), sTfR was higher ($\beta = 0.44$, $p = 0.2$, $n = 87$) and TBI was lower ($\beta = -0.06$ $p = 0.2$, $n = 87$)). This finding is consistent with recent data we have reported showing that p[Fe] in placentae from women carrying multiples was also significantly inversely associated with maternal iron (TBI) and hematologic status (Hb), indicating that women with increased iron demand had higher placental iron content (*Barad unpublished*). Similarly, in the multiples cohort we found that placental ERFE expression increased as p[Fe] (μg/g wet weight) increased ($\beta = 0.50$, $p = 0.004$, $n = 65$) and placental ERFE expression was inversely associated with maternal Hb concentration ($\beta = -0.21$, $p = 0.01$, $n = 110$) (**Figure 5.2**).

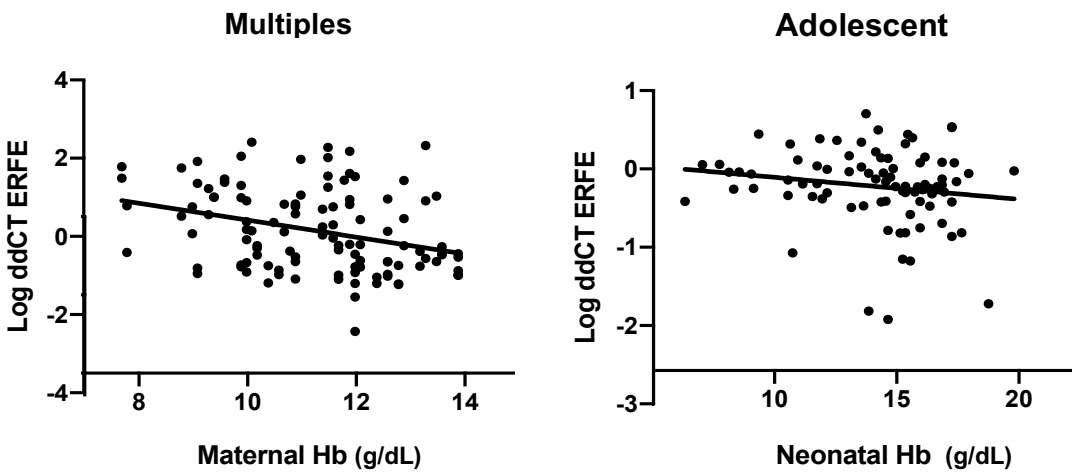

Figure 5.2: Placental ERFE expression as a function of maternal and neonatal hemoglobin

Placental Hormone Relationships in Adolescents Carrying Singletons

Relationships between EPO and ERFE mRNA expression in placentae from adolescents carrying single fetuses differed from associations observed in the multiples cohort. Placental EPO was significantly positively associated with maternal ERFE ($\beta = 0.43$, $p = 0.02$, $n = 92$) and Hb concentration ($\beta = 0.25$, $p = 0.03$, $n = 110$), although placental EPO was not significantly higher in anemic women ($p = 0.7$). In the adolescent cohort, placental ERFE expression demonstrated a stronger inverse association with neonatal Hb ($\beta = -0.04$, $p = 0.07$, $n = 77$) (**Figure 5.2**) and was not associated with maternal Hb ($\beta = -0.04$, $p = 0.42$, $n = 110$). Additionally, in the adolescent cohort placental ERFE was significantly positively associated with neonatal EPO ($\beta = 0.22$, $p = 0.002$) although this relationship appeared to be driven by GA ($\beta = 0.13$, $p = 0.06$). In the subset of adolescents with placental hepcidin mRNA expression evaluated ($n = 103$), hepcidin mRNA expression was not significantly associated with placental ERFE expression ($\beta = 0.2$, $p = 0.3$) or placental EPO

expression ($\beta = 0.02$, $p = 0.9$). Furthermore, maternal and neonatal ERFE were not associated with placental hepcidin mRNA expression.

Placental ERFE and EPO Concentration Differences Between Siblings

In the multiples cohort the mean difference in placental expression ($\Delta\Delta Ct$) between siblings was 1.98 [0.83 – 4.71] for ERFE and 108.9 for EPO [95%CI: 38.1 – 311.9]). The observed difference in newborn weight between siblings was not associated with the observed difference in placental ERFE expression ($p = 0.9$) or placental EPO expression ($p = 0.4$). Differences in ERFE or EPO expression between siblings were not significantly impacted by chorionicity or amnioncity . Differences in EPO and ERFE expression between siblings were not significantly impacted by whether the placenta was fused or discrete. Siblings exhibiting greater differences in placental EPO also had greater differences in placental ERFE ($p < 0.0001$).

Discussion

To our knowledge, this is the first study to measure placental ERFE and EPO mRNA expression and assess relationships between these measures and placental hormone concentrations, placental iron content and maternal and neonatal iron status at birth in two groups of newborns at higher risk of neonatal anemia and iron deficiency. Placental ERFE and EPO mRNA was detectable in all placentae, but these hormones were not associated with maternal, placental or neonatal iron status. Both EPO and ERFE were associated with Hb concentrations, but relationships differed by cohort. In placental tissue collected from newborns born to women carrying multiples, placental expression of EPO and ERFE were strongly inversely associated with maternal hematologic status, whereas in placentae obtained from newborns born to

adolescents carrying single fetuses, placental EPO transcript expression was positively associated with maternal Hb while placental ERFE expression was inversely associated with neonatal Hb concentrations.

The iron regulatory hormones EPO, ERFE and hepcidin are expressed within placental tissue as early as 25 weeks of gestation. Although in systemic circulation these hormones are all associated with iron status, in placental tissue, mRNA expression of these hormones was not significantly related to maternal or neonatal iron status, neonatal birth weight or placental weight. The lack of association between hormone expression and maternal, neonatal and placental iron status may be due to each hormone exhibiting functions other than regulating iron. Erythroferrone was discovered as an iron regulatory hormone in 2014 (31), but it was initially discovered several years earlier and found to function as a myokine involved in muscle lipid metabolism (40). EPO also appears to have non-erythropoietic roles and is involved in angiogenesis, wound healing, and has tissue protective properties (41, 42). Hepcidin is thought to be an anti-microbial peptide (8). Together these findings suggest that the additional functions of the hormones may take precedence over iron regulation in placental tissue.

The placenta is the sole interface responsible for iron transfer from mother to the developing fetus, but transfer to the fetus must be balanced against the iron requirements needed to support placental functions. Animal data has shown that the placenta sequesters iron for its own demands over those of the fetus a concept referred to as the 'selfish placenta' (43). Our data in the adult women carrying multiple fetuses supports this concept as placental iron content was higher in women with lower iron

and hematologic status (Barad). This finding is consistent with the finding of higher placental expression of EPO and ERFE in women with lower Hb concentrations. The selfish placenta concept did not apply in the adolescent cohort. In this group, placental iron content as well as EPO expression was higher in women with higher hemoglobin concentrations, and placental ERFE expression was more strongly associated with neonatal Hb concentrations. These findings suggest the selfish placental concept does not apply for all pregnancies.

Under hypoxic conditions, Erythroferrone is produced by erythroblasts in response to stimulation by EPO (31). No data exist on placental ERFE expression, this was the first study to demonstrate that placental expression of ERFE appears to increase when maternal or neonatal Hb is decreased. Whether maternal or neonatal Hb was the most strongly associated with placental ERFE expression differed between cohorts. Within the multiples cohort, maternal Hb concentration is more closely associated with placental ERFE expression. On the other hand, in the adolescent cohort, neonatal Hb is more closely associated with placental ERFE expression. These results further highlight that homeostatic mechanisms are not consistent throughout all women and placenta.

The placenta expresses EPO, and placental EPO expression has been found to increase under hypoxic conditions in sheep (14) and in newborn human twins (13). These findings are consistent with our observations in women carrying multiples, where maternal Hb was significantly inversely associated with placental EPO expression, suggesting low oxygen levels increase the placental expression of this hypoxia driven hormone. In the adolescent cohort, however, the inverse relationship

between maternal Hb and placental EPO was observed in the adolescents, again suggesting that placental functioning may differ in biologically immature gravida, as has been demonstrated in adolescent sheep models (44).

While the current paper presents the first data on placental ERFE expression, there are limitations that need to be considered. Evaluation of mRNA concentrations alone provides some evaluation of gene expression, but regulation of gene expression can occur post-transcriptionally and translationally. EPO appears to be regulated at the transcriptional and post-transcriptional level (45, 46) and only one study assesses muscle ERFE mRNA expression in relation to circulating serum concentrations, and these two appear to be correlated (40). We did not measure protein concentrations of these hormones in placental tissue which would have provided additional information on ERFE and EPO in placental tissue.

In conclusion, this was the first study demonstrating that the human placenta expresses erythropoietic and erythroferrone as early as 25 weeks of gestation. No strong consistent relationships were evident between placental ERFE or EPO transcripts and any of the measured markers of iron status in the mother, placenta or neonate. More work is needed in understanding the role of these hormones within placental tissue in various populations, as differences in homeostasis and partitioning exist between the adult women carrying multiple fetuses and pregnant adolescents.

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CHAPTER 6

UMBILICAL CORD SERUM FERRITIN CONCENTRATION IS INVERSLY ASSOCAITED WITH UMBILICAL CORD HEMOGLOBIN IN NEONATE BORN TO ADOLESCENTS CARRYING SINGLETONS AND WOMEN CARRYING MULTIPLES*

*Delaney KM, Guillet R, Fleming RE, Ru Y, Pressman EK, Vermeylen F, Nemeth E, O'Brien KO. Umbilical Cord Serum Ferritin Concentration is Inversely Associated with Umbilical Cord Hemoglobin in Neonates Born to Adolescents Carrying Singletons and Women Carrying Multiples. J Nutr 2019;149(3):406-15. doi: 10.1093/jn/nxy286.

Abstract

Background: It has been proposed that the fetus prioritizes iron for hemoglobin production over delivery to tissues. However, few studies have evaluated the interrelations between hemoglobin and multiple iron status biomarkers in umbilical cord blood. A full understanding is needed of how these parameters influence each other within cord blood to fully interpret iron and hematologic status at birth.

Objectives: We evaluated the determinants of neonatal hemoglobin and assessed the interrelations between hemoglobin, serum iron status indicators, and serum iron regulatory hormones in healthy neonates.

Methods: This was an observational study that assessed umbilical cord hemoglobin (Hb), serum ferritin (SF), erythropoietin (EPO), soluble transferrin receptor (sTfR), serum iron, hepcidin, vitamin B-12, folate, IL-6, and CRP measured in 234 neonates born to adolescents or to women carrying multiples. Correlations between these indicators were evaluated and mediation models consistent with the observed significant determinants of cord Hb concentrations were developed.

Results: A highly significant inverse association was found between cord SF and Hb concentrations that was not attributable to neonatal or maternal inflammation (as measured by IL-6 and CRP). The inverse association was present in the combined cohort, as well as in the adolescent and multiples cohorts independently. Mediation analyses found that EPO and hepcidin had significant indirect effects on cord Hb, associations that are explicable by mediation through SF and sTfR.

Conclusion: In contrast to observations made in older infants, a highly significant inverse association between Hb and SF, as well positive associations between Hb and

both sTfR and EPO, were observed in umbilical cord blood from neonates born to adolescents or women carrying multiples. These findings, combined with review of the published literature, indicate a need for analysis of the relations between multiple parameters to assess iron and hematologic status at birth.

Introduction

Iron (Fe) is an essential nutrient involved in numerous metabolic processes such as oxygen transport, mitochondrial function, as well as growth and development (1). Iron deficiency during fetal and early life developmental periods has been associated with multiple adverse neurodevelopmental and cognitive outcomes (2-6). The fetus relies on maternal Fe stores during pregnancy, and by late gestation 5-8 mg of Fe per day are transported across the placenta to support fetal demands. When Fe availability in utero is limited, the human fetus has been shown to prioritize the Fe demands of erythropoiesis over other tissue demands, including the brain (7, 8). In utero, hemoglobin (Hb) concentrations are elevated compared to values any time thereafter in response to a relatively hypoxemic environment (9). After birth, Fe that was once in erythrocyte heme becomes redistributed and utilized by other tissues or stored as ferritin. As such, there are dynamic changes in biomarkers of Fe status across early infancy (10).

Normative data on Hb concentrations across a range of gestational ages at birth and over the first few weeks of life have been reported in large cohorts of neonates (9, 11). However, data evaluating Fe status indicators in umbilical cord blood have been compiled from much smaller study populations and many have evaluated outcomes assuming that associations between Hb and Fe status indicators in cord blood mirror those found in older infant, pediatric and adult populations. Few studies however have tested these assumptions or assessed relationships between Fe status indicators and Hb or erythropoietic regulatory hormones in cord blood. In particular, serum ferritin (SF) is commonly utilized as a biomarker of body Fe stores but the vast majority (>80%) of

identified studies that published both Hb and SF data in cord blood did not report any data on possible associations between these two biomarkers (**Appendix: Table 1**). Recent studies have also raised concerns with the interpretation of SF in older pediatric and pregnant cohorts and have highlighted the need to evaluate this indicator in relation to markers of inflammation and to established iron status biomarkers (12, 13).

We recently completed two studies evaluating neonatal Fe status indicators in umbilical cord blood in a group of 234 neonates born to two obstetric groups known to be at higher risk for maternal Fe deficiency and anemia (14, 15). In each of these neonatal cohorts, Hb concentrations, Fe status indicators, Fe regulatory hormones and inflammatory markers were evaluated. In both cohorts, an unexpected and highly significant inverse association between SF and Hb concentrations was evident (14, 16). The main goal of this analysis was to investigate the interrelationships between Hb concentrations in umbilical cord blood and a more comprehensive panel of Fe status indicators and Fe regulatory hormones across late gestation. A secondary goal was to compare our data to other published findings on Hb and SF in umbilical cord blood to determine if our SF and Hb findings were unique to our particular neonatal cohorts, or if similar findings have been reported in other neonatal study populations.

Methods

Participants

Pregnant women were recruited from Strong Memorial Hospital and Highland Hospital in Rochester, NY as previously reported (15, 17). Informed written consent was obtained from all participants > 14 y of age, and parental consent and adolescent

assent were obtained from adolescents ≤ 14 y of age. The total study population consisted of 374 neonates born to two cohorts of higher-risk, but otherwise healthy gravida. Neonates had Fe status indicators measured within cord blood at birth and were excluded if they did not have both Hb and SF measurements within cord blood, resulting in a study population of 234 neonates. Therefore, the final study population was comprised of 108 neonates born to 64 women carrying multiples (age ≥ 21) and 126 singleton neonates born to pregnant adolescents (age 13-18 y) (**Figure 6.1**). All studies were approved by the institutional review boards of the University of Rochester and Cornell University. Gestational age (GA) for both populations were determined using standard criteria or using the known date of in vitro fertilization (IVF) in the multiples cohort. Preterm delivery was defined as birth occurring before 37 weeks of gestation, while early term delivery was defined as birth between 37-38 weeks of gestation. Low birth weight (LBW) was defined as birth weight < 2,500 g. In all women, a baseline health history was obtained and pertinent maternal and neonatal data at birth were abstracted from medical records. Maternal Fe status data from the adolescent (14, 17, 18), and multiples cohort (15), and descriptive data on Fe status indicators in the neonates (15, 16), have been published.

Serum collection and biochemical analyses

Umbilical cord blood (~15 mL) was obtained at delivery. In both cohorts, whole blood was sent to the University of Rochester core laboratory for assessment of Hb concentrations using a Cell-Dyn 4000 hematology analyzer (Abbott diagnostics, Santa Clara, CA). The remaining blood samples were centrifuged, separated and stored at -80 until analysis. Cord blood was not obtained from 50 newborns in the adolescent

cohort due to lack of study personnel at delivery (n = 9), delivery at a different hospital (n = 8), fetal death *in utero* (n = 5), maternal refusal (n = 3) and other miscellaneous reasons (n = 25). Neonatal mortality rate in the multiples cohort was 1.6% (3 deaths per 186 live births). Neonatal anemia was defined using standard definitions as a cord Hb concentration < 130 g/L (19). Serum ferritin (SF), soluble transferrin receptor (sTfR) and hepcidin were measured by ELISA (Ramco Laboratories, Stafford, TX) as previously described (14, 15). The SF coefficient of variation (CV) of control samples provided in the kit fell within specifications for all kits and averaged 8% for the multiples cohort and 4% for the adolescent cohort. Cord serum Fe was measured by atomic absorption spectrophotometry (Perkin Elmer AAnalyst 800, Waltham, MA). Cord erythropoietin (EPO) values were measured by immunoassay (Siemens Immulite 2000, Erlangen, Germany). Hepcidin, CRP and IL-6 were measured with two different assays between cohorts. Within the multiples cohort, hepcidin, CRP and IL-6 was measured with ELISA as previously described (15). Within the adolescent cohort, hepcidin was measured with an ELISA and IL-6 and CRP were measured using a commercial immunoassay as previously described (14). Sample sizes vary per indicator due to insufficient sample volume to analyze all indicators.

Statistical Analysis

Subject characteristics and Fe status indicators were compared between cohorts using two-tailed t-tests or Chi-squared test. Indicators that were analyzed with different assays between cohorts (CRP, IL-6 and hepcidin) were standardized by dividing each measurement by its respective cohort's mean and dividing this number by the standard

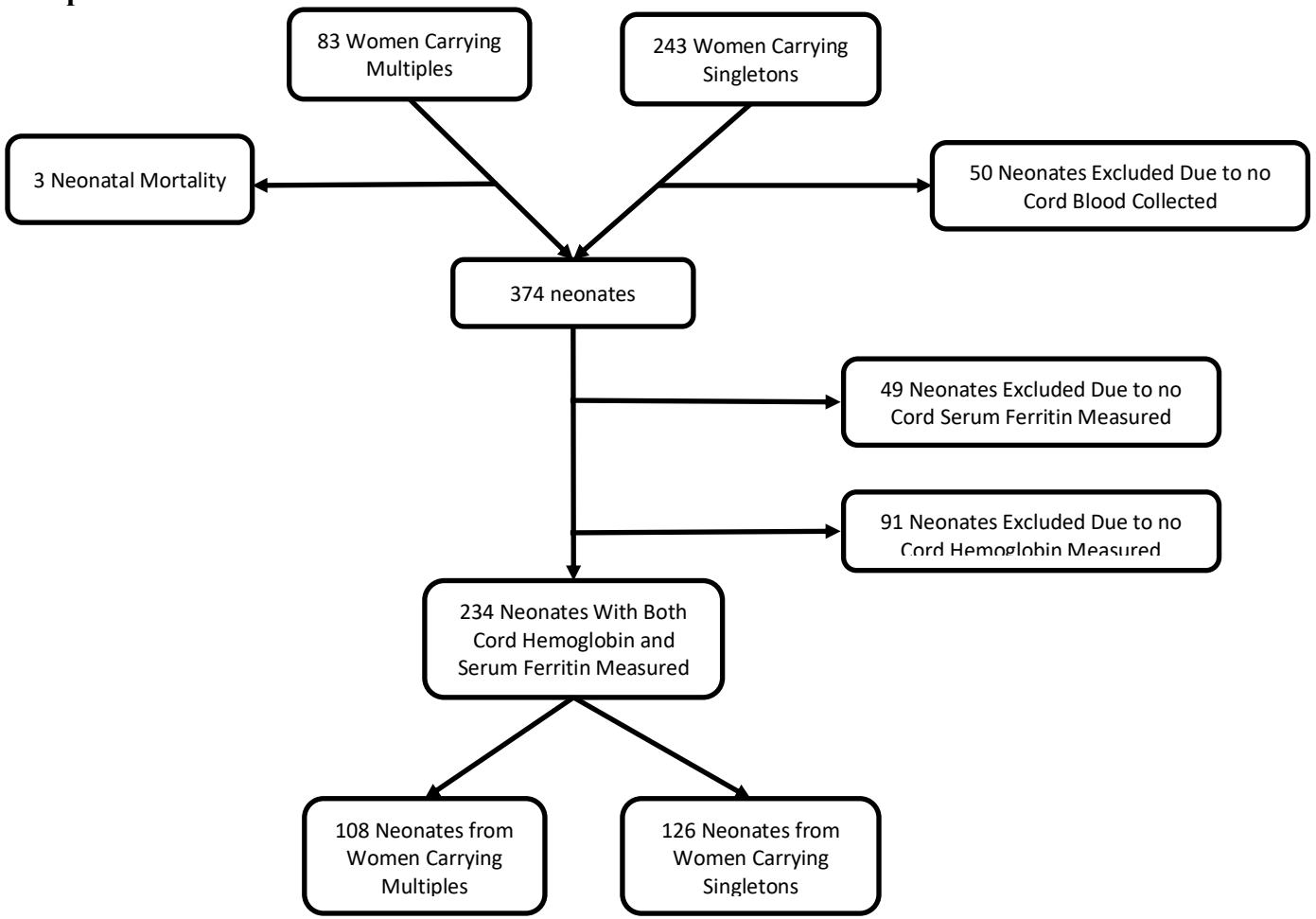
deviation of the cohort. Pairwise and partial Pearson correlations were assessed between Fe status indicators, inflammatory markers and Hb. Neonates were then divided into tertiles based on their Hb status. Iron status indicators and regulatory hormones were evaluated by tertile. Differences in iron status indicators by Hb tertiles were compared using a general linear model controlling for birth weight. This was followed by Tukey honest significant difference (HSD) analyses for multiple comparisons. Multiple regression was utilized to determine predictors of Hb status. Variables were tested simultaneously and eliminated by backward selection until only statistically significant predictors remained. All analyses were performed using the multiple mothers ID number as a random coefficient to control for lack of independence in the multiples cohort. The previous statistical analyses were performed using JMP 12.0 (SAS Institute Inc, Cary, NC). To assess interrelationships between Fe status indicators and Hb a mediation model was developed using STATA 15 (StataCorp LLC, College Station, TX). To determine the amount of variance captured by the model, an R^2 for linear mixed models was calculated following published methods (20). Results of statistical tests were considered significant at p-values < 0.05.

Literature Search

A PUBMED and Web of Science search for articles written in English was used to identify studies that measured both SF and Hb in cord blood. The Web of Science search was as follows: ((Hemoglobin or Anemia) AND ("serum ferritin" or ferritins) AND ("cord blood" or "fetal blood")). The PUBMED search was as follows: (Hemoglobins[Mesh] OR Hematocrit[Mesh] OR Anemia, Iron Deficiency[Mesh] OR

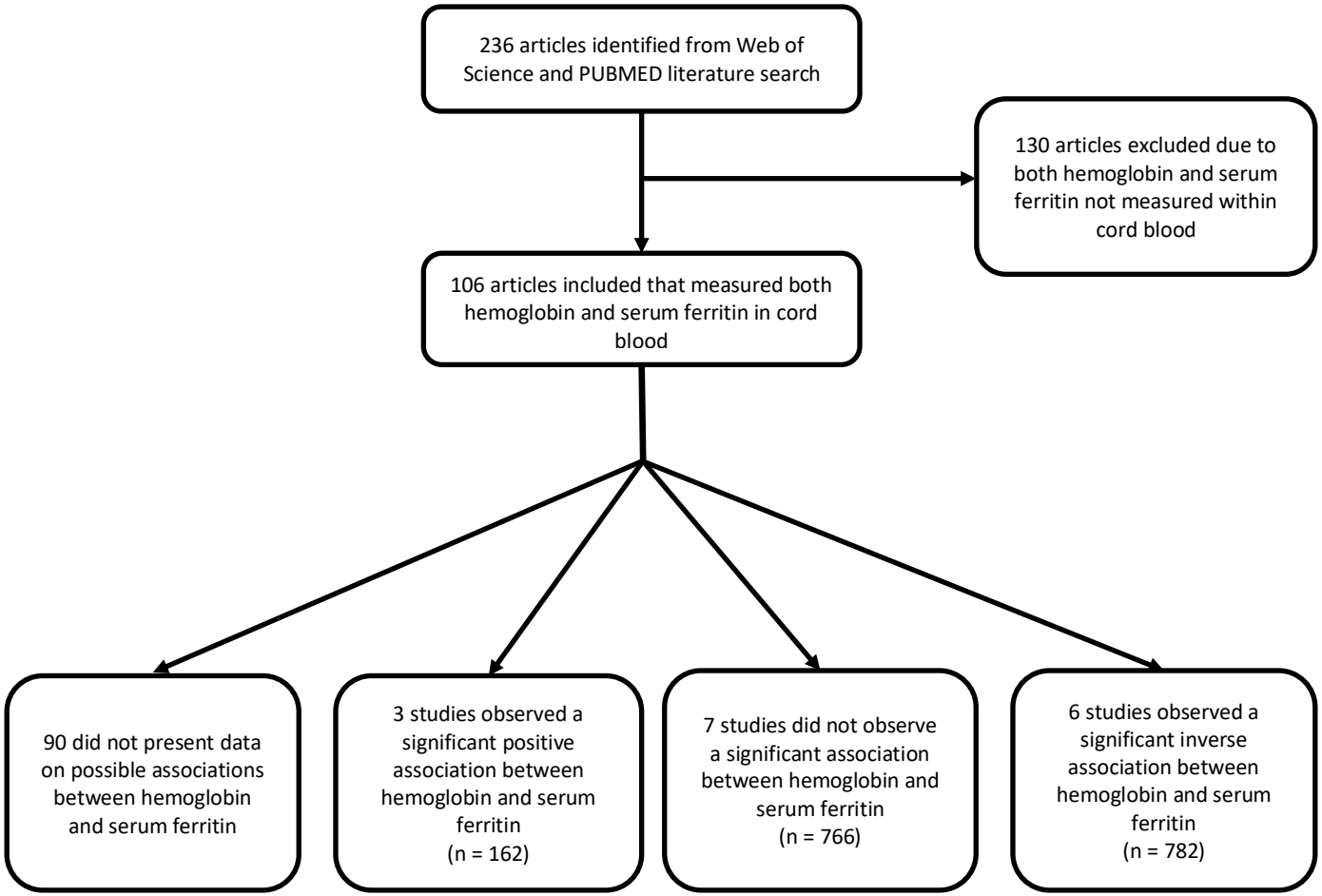
Hemoglobin*[tiab] OR haemoglobin*[tiab] OR hematocrit*[tiab] OR iron deficiency[tiab]) AND (Ferritins[Mesh] OR ferritin*[tiab]) AND (Fetal blood[Mesh] OR fetal blood[tiab] OR cord blood[tiab]). **Figure 6.2** demonstrates the results from this literature search and a list of main findings from the 106 identified studies can be found in **Appendix Table 1**.

Figure 6.1: Participant Flow Chart



Flowchart of participants recruited to Strong Memorial Hospital and Highland Hospital and inclusion into study by umbilical cord iron status indicator measurements.

Figure 6.2: Literature Search on Hemoglobin and Serum Ferritin in Umbilical Cord Blood



Flowchart of all identified articles from both PUBMED and Web of Science and those included when both serum ferritin and hemoglobin were measured in umbilical cord blood.

Results

Neonatal Characteristics

Neonatal characteristics are summarized in **Table 6.1**. Maternal characteristics, background data, recruitment procedures, and loss to follow-up have been described in detail elsewhere (15, 17, 21). The mean number of offspring within the multiples cohort was two newborns. Consistent with national data in women carrying multiples (22-24), 65% of neonates born to women carrying multiple fetuses were born preterm. One fourth of these preterm births were due to pregnancy complications that have been previously reported (16). As expected (24), the majority (71%) of multiple birth neonates were born LBW and 92% of these LBW neonates were those born prematurely. Although pregnant adolescents typically have a higher risk of poor pregnancy outcomes (25), only 2% of babies born to this adolescent population were LBW and none were born prematurely. There was a higher prevalence of African American females within the adolescent cohort, consistent with national data demonstrating adolescent pregnancy disproportionately impacts minorities (24). Within the multiples cohort the majority of neonates (74%) were delivered via C-section, consistent with national data (26). However, only 10% of neonates within the adolescent cohort were delivered via C-section. There was a higher prevalence of female neonates within the multiples cohort, a finding that was consistent with published data documenting an increased prevalence of female births in women carrying multiples (27-30).

Table 6.1: Neonatal Characteristics

Variable	Whole Population (*n*=234)	Multiples Cohort (*n*=126)	Adolescent Cohort (*n*=108)
Gestational Age (weeks)	37.07 ± 3.36	34.70 ± 2.73[a]	39.84 ± 1.22[b]
Pre-term (< 37 weeks) %	35	65	0*
Early Term (37-38 weeks) %	30	35	24*
Birth weight (kg)	2.70 ± 0.7	2.22 ± 0.5	3.28 ± 0.4*
Low Birth Weight (<2.5kg) %	39	71	2*
Very Low Birth Weight (<1.5kg) %	04	07	00*
Race			
African-American, %	46	21	75*
Ethnicity			
Hispanic, %	17	09	26*
Mode of Delivery			
Cesarean section, %	45	74	10*
M/F ratio	0.97	0.88	1.08

[1]Data are presented as arithmetic mean ± SD, or percentage. [2] Indicates statistical difference from Multiples cohort ($p<0.05$).

Neonatal Iron Status Indicators

Data on Hb, prevalence of anemia, neonatal Fe status indicators and inflammatory markers are presented in **Table 6.2.** There was a significant difference in cord Hb concentrations between the multiples and adolescent cohorts (153.5 g/L (*n* = 126) versus 143.2 g/L (*n* = 108), p = 0.03) and within the entire cohort Hb was negatively associated with gestational age (β = -0.12, p = 0.04, *n* = 234). Other biomarkers that were significantly negatively associated with gestational age were serum Fe (β = -0.04, p = 0.001, n=224), CRP (β = -0.38, p < 0.001, *n* = 172) and folate

(β = -1.09, p = 0.002, *n* = 158). Biomarkers that were significantly positively associated with gestational age were sTfR (β = 0.05, p < 0.001, *n* = 234), and EPO (β = 0.13, p < 0.001, *n* = 218). The biomarkers that were not associated with gestational age included: SF (β = 0.024, p = 0.16, *n* = 234), hepcidin (β = 0.02, p = 0.32, *n* = 233), vitamin B-12 (β = 0.01, p = 0.37, *n* = 150) and IL-6 (β = 0.03, p = 0.11, *n* = 217).

Associations between Fe status indicators and regulatory hormones

Pairwise correlations for all Fe status indicators and regulatory hormones can be seen in **Table 6.3**. Within each individual cohort (multiples; p < 0.0001, *n* = 108 and adolescent; p = 0.004, *n* = 126) and in the combined population (p < 0.0001, *n* = 234) a highly significant inverse association between SF and Hb was observed (**Figure 6.3).** This inverse association remained significant after controlling for maternal and neonatal inflammation using either cord IL-6 (p < 0.01, *n* = 217), cord CRP (p < 0.01, *n* = 172) or both (p < 0.01, *n* = 165). In addition, CRP and IL-6 concentrations did not significantly differ in neonates within the highest and lowest Hb tertiles (**Table 6.4**). The inverse association did not appear to be driven by the high proportion of anemic neonates within this study population, as the inverse association remained significant when only the non-anemic neonates were evaluated (β = -0.63, p < 0.001, *n* = 195). Moreover, the negative associations between cord SF and Hb was independent of neonatal race, ethnicity, mode of delivery, gestational age, maternal inflammation or maternal ppBMI.

Table 6.2: Umbilical Cord Red Blood Cell Indices, Serum Iron Status Indicators and Iron Regulatory Hormones

Variable	*n*	Whole Population	*n*	Multiples Cohort	*n*	Adolescent Cohort
Hemoglobin (g/L)	234	147.6 ± 1.80	126	153.4 ± 2.30	108	143.2 ± 2.50*
Anemia, %	39	17	15	12	24	22
Serum Ferritin (μg/L)	234	110.82 (1.06)	126	103.28 (1.09)	108	120.31 (1.07)
Serum Iron (mg/L)	224	2.64 (1.04)	123	2.97 (1.06)	101	2.28 (1.05)*
Serum EPO (mIU/mL)	218	22.40 (1.07)	121	16.64 (1.10)	97	32.45 (1.09)*
Serum TfR (mg/L)	234	6.51 (1.03)	126	5.86 (1.05)	108	7.38 (1.04)*
Standardized Serum Hepcidin	-	-	126	0.38 (0.99)	107	-0.03 (0.09)
Serum Hepcidin (ng/mL)			126	13.78 (1.10)	107	92.13 ± 1.09
Standardized Serum IL-6	-	-	121	0.50 (1.11)	96	0.67 ± 1.06
Serum IL-6 (pg/mL)			121	3.81 (1.18)	96	10.22 (1.15)
Standardized Serum CRP	-	-	118	0.05 (1.08)	54	0.002 (1.33)
Serum CRP (mg/L)			118	0.10 (1.10)	54	0.28 (1.13)
Undetectable CRP (%)			80	68	43	80
MCV	191	105.96 ± 0.58	123	107.76 ± 0.74	68	102.72 ± 0.81*
MCH	183	35.76 ± 0.21	117	36.44 ± 0.26	66	34.55 ± 0.28*
MCHC	183	33.75 ± 0.08	116	33.82 ± 0.09	67	33.66 ± 0.14
Serum Vitamin B-12 (pg/mL)	150	717.5 (1.05)	101	699.79 (1.07)	49	755.44 (1.07)*
Serum Folate (nmol/L)	150	35.25 ± 1.11	112	38.45 ± 1.50	46	31.51 ± 1.48*

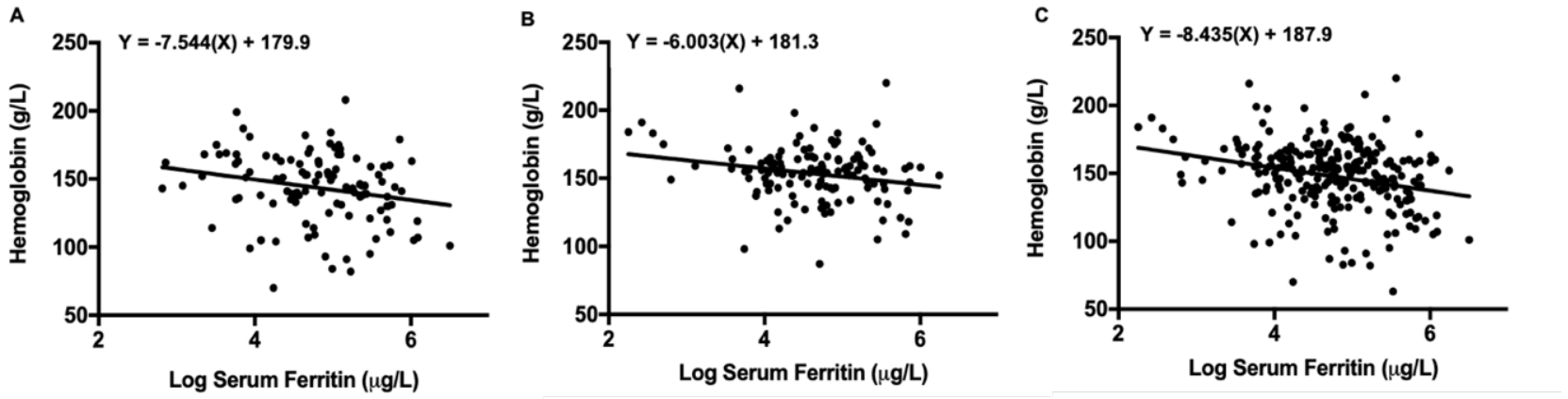
Values are arithmetic means ± SE and geometric mean (SE). *Indicates significant difference from Multiples cohort ($p<0.05$).

Table 6.3: Correlations Between Iron Status Indicators, Regulatory Hormones and Hemoglobin in Umbilical Cord Blood

	Hb (g/L) (n = 233-234)	SF (µg/L) (n = 234)	sTfR (mg/L) (n = 49-234)	Serum Fe (mg/L) (n = 48-224)	EPO (mIU/mL) (n = 49-218)	Std Hep (n = 217-234)	Std IL-6 (n = 213-217)	Vitamin B-12 (pg/mL) (n = 149 - 150)	Folate (nmol/L) (n = 145 - 150)	Std CRP (n = 37 - 47)
Hb (g/L)	1.0000	**-0.2610***	**0.1724***	**0.1452***	-0.0291	**-0.1401***	0.0378	0.0833	**0.2026***	-0.2543
SF (µg/L)		1.0000	**-0.1318***	**0.1328***	**-0.1512***	**0.4288***	0.0344	**0.1714***	0.0869	-0.0804
sTfR (mg/L)			1.0000	-0.0464	**0.4457***	**-0.2265***	0.1282	0.1174	-0.1376	-0.0962
Serum Fe (mg/L)	0.0987			1.0000	-0.0225	-0.0078	0.0586	0.0369	**0.3282***	**-0.3817***
Serum EPO (mIU/mL)					1.0000	-0.0877	**0.2666***	0.1307	**-0.1776***	**0.4240***
Std Serum Hep	-0.1201				**-0.1367***	1.0000	**0.1773***	**0.2014***	0.0512	**0.3680***
Std Serum IL-6							1.0000	0.0000	0.0842	**0.0.4511***
Serum Vitamin B-12 (pg/mL)								1.0000	0.1209	-0.1684
Serum Folate (nmol/L)									1.0000	-0.3007
Std CRP			**-0.4572***	-0.1552	-0.0044					1.0000

[1]Values are pearson pairwise correlation coefficients (r) for relationships between serum iron status indicators, iron regulatory hormones and hemoglobin in umbilical cord blood from neonates born to pregnant adolescents and women carrying multiples are shown above the diagonal. Partial correlation coefficients (partial r) for relationships between serum iron status indicators, iron regulatory hormones and hemoglobin in umbilical cord blood from neonates born to pregnant adolescents and women carrying multiples, while controlling for birth weight and using the mothers ID as a random effect, are shown below the diagonal. *Indicates $p < 0.05$.

Figure 6.3: Inverse Association Between Umbilical Cord Hemoglobin and Serum Ferritin



(A) Neonates in the multiples cohort. (B) Neonates in the adolescent cohort. (C) All neonates as the combined cohort.

Table 6.4: Umbilical Cord Iron Status Indicator Means by Hemoglobin Tertiles

	n	Tertile 1	*n*	Tertile 2	*n*	Tertile 3	p-value
Hb (g/L)	60	11.87 (11.57, 12.17)[a]	125	15.22 (15.01, 15.43)[b]	49	17.69 (17.39, 18.02)[c]	<0.01
SF (µg/L)	60	148.90 [123.5, 179.50][a]	125	108.50 [94.77, 124.23][b]	49	78.44 [64.44, 95.48] [c]	<0.01
sTfR (mg/L)	60	5.93 [5.34, 6.59] [a]	125	6.43 [5.96, 6.93][a]	49	8.17 [7.31, 9.13][b]	<0.01
BW (g)	60	3034.40 (2885.77, 3183.02)[a]	125	2876.18 (2757.51, 2994.86)[ab]	49	2715.05 (2603.97, 2898.13)[b]	<0.01
African American (%)	37	62	56	45	15	31	<0.01
Serum EPO (mIU/mL)	55	23.45 [18.76, 29.31][ab]	118	20.77 [17.70, 24.36][b]	45	30.5 [24.01, 38.87][a]	0.01
GA (wk)	60	37.19 (36.78, 37.60)	125	37.33 (37.03, 37.64)	49	37.77 (37.37, 38.17)	0.10
Serum Fe (mg/L)	57	2.59 [2.26, 2.99]	121	2.56 [2.32, 2.83]	46	2.96 [2.53, 3.45]	0.15
Std Serum IL-6	53	0.05 [-0.25, 0.34]	118	-0.08 [-0.28, 0.13]	46	0.21 [-0.10, 0.52]	0.33
Serum Folate (nmol/L)	36	34.19 (30.49, 37.90)	94	36.10 (33.53, 38.68)	28	35.84 (32.09, 39.58)	0.33
Male (%)	32	53	55	45	28	57	0.37
Serum Vitamin B-12 (pg/mL)	33	714.23 [599.07, 851.59]	90	695.25 [616.82, 783.66]	27	826.05 [689.00, 990.36]	0.40
Std Serum Hep	60	0.20 [-0.05, 0.45]	124	-0.08 [-0.26, 0.10]	49	-0.15 [-0.42, 0.13]	0.44

[1]Data are presented as arithmetic mean (95% CI) or geometric mean [95% CI]. All means were determined controlling for birth weight. Values within a row that do not share a superscript demonstrate statistical differences between serum ferritin tertiles ($p<0.05$). Undetectable values were excluded from calculations. CRP, c-reactive protein; EPO, erythropoietin; Fe, iron; Hb, hemoglobin; Hep, hepcidin; IL-6, interleukin-6; SF, serum ferritin; sTfR, soluble transferrin receptor; std, standardized.

Impact of Fe status and regulatory hormones on Hb status

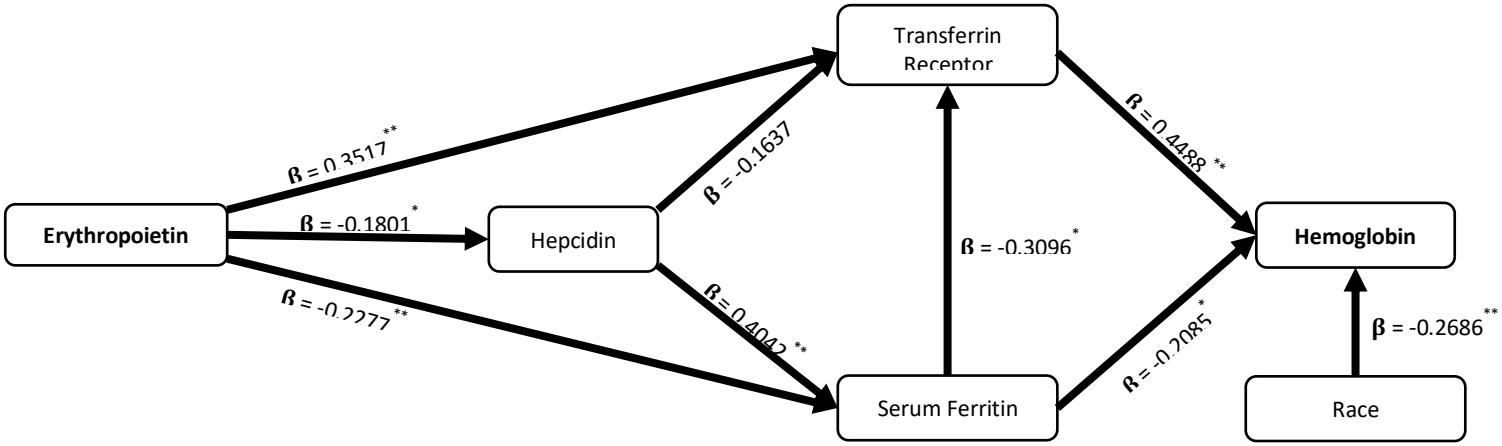
Multiple regression analysis revealed that neonatal determinants of Hb status in the combined cohort were BW, SF, sTfR, and race. Together these four variables accounted for 22% of the variance in cord Hb (**Table 6.5**). To further explore interrelationships between study variables and their impact on Hb status, a mediation model was developed (**Figure 6.4**). Using standardized variables within this approach, the strongest associations were evident between cord Hb and sTfR concentrations (β = 0.45; $p < 0.001$, $n = 221$), and between cord Hb and SF concentrations (β= -0.21; $p < 0.01$, $n = 221$). Hepcidin exhibited significant direct associations with SF, and EPO exhibited significant direct associations with SF and sTfR. Both regulatory hormones exhibited indirect associations with Hb (**Table 6.6**). The mediation model explained 23% of the variance found in Hb status.

Table 6.5: Determinants of Umbilical Cord Hemoglobin

Hemoglobin	Coefficient	Standard Error	p-value
Intercept	16.931	1.330	< 0.01
Log Serum Ferritin (µg/L)	-0.764	0.189	< 0.01
Log Serum Transferrin Receptor (mg/L)	1.582	0.332	< 0.01
Race	0.597	0.178	<0.01
Birth Weight (g)	-0.001	0.0002	0.03

[1]Multivariate analysis with hemoglobin as the response. Hb, hemoglobin; EPO, erythropoietin; sTfR, soluble transferrin receptor; SF, serum ferritin.

Figure 6.4: Direct Effects of Umbilical Cord Iron Status Indicators and Regulatory Hormones on Hemoglobin Status



A mediation model was developed to evaluate the direct and indirect effects of these iron status indicators and regulatory hormones on hemoglobin status Superscripts indicate * ($p<0.05$) and ** ($p<0.001$). Direct pathways are shown with a solid line. Indirect pathways are shown in the table.

Table 6.6: Indirect Effects of Umbilical Cord Iron Status Indicators and Regulatory Hormones on Hemoglobin Status

Pathway	Coefficient	Std Error	p-value
EPO-TfR-Hb	0.1578	0.051	0.002
Hep-SF-Hb	-0.0843	0.029	0.004
EPO-SF-Hb	0.0475	0.020	0.018
SF-TfR-Hb	-0.1389	0.073	0.057
Hep-SF-TfR-Hb	-0.0562	0.030	0.063
EPO-Hep-SF-Hb	0.0152	0.010	0.064
EPO-SF-TfR-Hb	0.03164	0.018	0.075
Hep-TfR-Hb	-0.0735	0.048	0.123
EPO-Hep-SF-TfR-Hb	0.0101	0.010	0.145
EPO-Hep-TfR-Hb	0.0132	0.010	0.177

Indirect effects on hemoglobin from the mediation model in Figure 6.4.

Literature Search on Hemoglobin and Ferritin in Cord Blood

The literature search identified 106 studies that reported data on both Hb and SF in umbilical cord blood (**Appendix Table 1**). In this relatively large body of literature, only 16 studies reported any information on possible associations between these two indicators. The results of these findings can be seen in Figure 2 and Supplemental Table 1. Six of these 16 studies (38%) reported a significant inverse association between Hb and SF. Five of the five studies that found an inverse association were undertaken in healthy term neonates born to both anemic and non-anemic mothers (14, 31-34), and the other was a study of neonates born to diabetic mothers (35). Two of the 15 studies (20%) reported a positive association between Hb and SF; these studies were undertaken in 135 total neonates born to women with Fe deficiency anemia (n = 100) and non-anemic women (n = 35) (36, 37). One additional study also reported a positive association, but this study evaluated percutaneous umbilical cord sampling over a wide gestational range during pregnancy, which might not reflect status within cord blood at birth (38). The remaining 7 of the 15 identified studies assessed the possible relationship between Hb and SF but found that it was not significant. All 7 of these study cohorts were comprised of healthy term neonates (sample sizes ranging from 44 – 193 neonates) (39-45). In addition, while only 15 of the studies identified in the literature review reported data on the correlation between Hb and SF, one additional study (**Appendix Table 1**) in a group of 300 neonates born to anemic mothers noted that neonates with anemia (assessed within cord blood) exhibited higher SF concentrations (46).

Discussion

The present study uniquely evaluated Hb, serum Fe status indicators, serum Fe regulatory hormones and inflammatory markers in umbilical cord blood obtained from a large cohort of neonates. Neonates studied were born to women at higher risk of Fe deficiency, providing opportunities to evaluate interactions between these biomarkers when iron availability across gestation may have been constrained. Similarly, the increased risk of preterm birth in women carrying multiples provided an opportunity to evaluate the possible impact of gestational age on observed associations. A highly significant inverse association between cord Hb and cord SF was evident in these neonates, a finding that was not driven by maternal or neonatal inflammation (CRP or IL-6), or influenced by the gestational age of the neonate at birth. Moreover, a comprehensive literature search on cord Hb and SF lends support to our findings. Our results highlight challenges inherent with the interpretation of Fe status biomarkers in umbilical cord blood and the need to obtain normative data on Fe status at birth in larger cohorts of neonates.

Nearly 20% of neonates studied were anemic and 40-50% of the women that gave birth to these neonates were anemic at delivery (14, 15). The prevalence of anemia was significantly higher among African American neonates and among their African American mothers. Normative data on possible racial differences in neonatal Hb concentrations are lacking. Currently, the Institute of Medicine recommends lowering the Hb cutoff for African American women by 8.0 g/L and for African-American children under 5 years of age by 4.0 g/L, although the CDC does not recommend race-specific cutoffs for children under 5 years of age (47, 48). National

Hb data from newborns are not available as The National Health and Nutrition Examination Survey (NHANES) does not include individuals under 1 year of age (49). Currently neonatal Hb concentrations are not routinely monitored at birth unless the newborn is identified as "at-risk". The American Academy of Pediatrics (AAP) has defined "at-risk" neonates as those that are born prematurely, low birth weight, or born to women of low socio-economic status (50).

The majority of Fe found in the human neonate at birth (70%) is present within the red blood cell (RBC) compartment (51). Newborn autopsy studies have estimated that every 1 μg/L of SF represents 2.7 mg/kg of storage Fe (31), and that in the presence of fetal hypoxia, Fe utilization is prioritized for erythropoietic demands at the expense of tissue and storage Fe (8, 52, 53). The exact amount of Fe stored within SF in umbilical cord blood is unknown (54). Within our cohort we observed a highly significant, inverse association between Hb and SF concentrations, such that neonates with the lowest Hb status exhibited the highest SF concentrations. Other studies with data on these biomarkers in cord blood support this inverse association between Hb and SF and have attributed this finding to a preferential use of Fe in support of erythropoietic demands (14, 31-35). This interpretation however, does not explain the presence of elevated umbilical cord ferritin concentrations in neonates with the lowest Hb concentrations.

The unexpected association between low cord Hb and elevated cord SF concentrations highlights what appears to be an inability of some neonates within this cohort to utilize iron stored within SF for RBC production, a finding that did not appear to be driven by inflammation as detailed above. In addition to Fe, folate and

vitamin B-12 deficiencies have also been associated with anemia, but this cohort of neonates were not deficient in either vitamin using standard cut-offs (55, 56). Red blood cell production is also influenced by other nutrients that were not evaluated in this cohort, including vitamin D, zinc, selenium, copper and vitamin A (57-59). When compared to adult erythrocytes, newborn erythrocytes exhibit markedly different metabolic characteristics, morphologies and membrane composition that may alter the ability to use storage Fe for Hb production in utero (9).

Hepcidin, EPO and erythroferrone are regulatory hormones that play a role in Fe metabolism and RBC production (60). Hepcidin limits Fe export from the enterocyte and decreases release from body stores during conditions of Fe sufficiency or inflammation. Hepcidin's role in regulating fetal Fe accretion is increasingly recognized (16, 61), but findings relating hepcidin to Hb concentrations in cord blood are mixed. In our cohort, significant negative direct and indirect associations were observed between Hb and hepcidin. This observation differs from two recent studies in 291 term (62, 63), and 121 preterm neonates (63), both of which found no significant correlation between these variables. Additionally, a small study of 45 newborns born to anemic (n=30) or non-anemic (n=15) mothers found a positive association between Hb and hepcidin (36). However, none of these studies included concurrent measures of a full panel of Fe regulatory hormones or inflammatory markers that may also be influencing study outcomes.

Erythropoietin is an erythropoietic hormone that increases Fe utilization in support of RBC production under conditions of hypoxia. Studies assessing cord blood EPO concentrations in neonates under hypoxic in-utero conditions (including pre-

eclampsia, placental dysfunction, maternal smokers or IUGR) found that cord EPO concentrations were elevated compared to neonates under non-hypoxic conditions (64-67). Only one of these studies concurrently measured Hb concentrations and found that neonates born to mothers who smoked during pregnancy had higher EPO and Hb concentrations compared to non-smokers (67). Within our cohort no direct association between EPO and Hb was observed, but a significant positive indirect association was mediated through EPO's association with SF, sTfR and hepcidin. Serum transferrin receptor concentrations can be used as a marker of iron restrictive erythropoiesis, but it is important to note that within newborns and infants this marker is also reflective of increased erythropoietic activity. The positive association between EPO and Hb was unexpected, since low Hb concentrations would be expected to stimulate EPO production as seen in adults (68) and neonates with severe anemia (hemolytic anemia and Rh immunization) (69, 70). However, other studies in healthy term neonates also reported a lack of significant direct association between Hb and EPO, although these study cohorts only included non-anemic neonates (71, 72).

Erythroferrone is a newly identified Fe regulatory hormone, which is stimulated by EPO to reduce hepcidin expression (60). A limitation to the current study is that no erythroferrone measurements were obtained due to lack of an available validated human assay when these studies were undertaken. Of note, our current mediation analysis found a direct negative relationship between umbilical cord EPO and hepcidin concentrations, an association which has found to be mediated by erythroferrone in other studies (60, 73-75). More data are needed on neonatal

erythroferrone concentrations to determine if this marker has additional diagnostic utility at this life stage.

The present observations in a group of newborns at increased risk for anemia demonstrated unexpected relationships between common Fe status biomarkers and regulators of Fe homeostasis and erythropoiesis. Until normative umbilical cord blood data on these indicators are available, care should be taken when applying assumptions based on observations made in older infants or adults. Our findings highlight concerns with the use of SF as an index of neonatal Fe sufficiency. Elevated concentrations of SF in cord blood were more common among anemic newborns, which may indicate that other factors (e.g. inflammation, EPO insensitivity) may limit use of this pool of iron for erythropoiesis. Improvements in interpretation of these biomarkers at birth is needed to identify neonates with insufficient Fe stores given the increasing links between suboptimal Fe status and subsequent adverse neurodevelopmental outcomes.

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CHAPTER 7

UMBILICAL CORD ERYTHROFERRONE IS INVERSLY ASSOCAITED WITH HEPCIDIN, BUT DOES NOT CAPTURE THE MOST VARIABILITY IN IRON STATUS OF NEONATES BORN TO TEENS CARRYING SINGLETONS AND WOMEN CARRYING MULTIPLES*

*Delaney, KM; Guillet, R; Pressman, E.K; Ganz, T; Nemeth, E; O'Brien, K.O. Umbilical cord erythroferrone is inversely associated with hepcidin, but does not capture the most variability in iron status of neonates born to teens carrying singletons and women carrying multiples, J Nutr. 2021, *in press*

Abstract

Background: The developing fetus requires adequate iron and produces its own hormones to regulate this process. Erythroferrone (ERFE) is a recently identified iron regulatory hormone, and normative data on ERFE concentrations and relationships between iron status as well as other iron regulatory hormones at birth are needed.

Objective: The objective of this study was to characterize cord ERFE concentrations at birth and assess interrelationships between ERFE, iron regulatory hormones and iron status biomarkers in two cohorts of newborns at higher risk of neonatal anemia.

Methods: Umbilical cord ERFE concentrations were measured in extant serum samples collected from neonates born to women carrying multiples (age: 21 – 43 y; n = 127) or teens (age: 14 – 19 y; n = 164). Relationships between cord blood ERFE and other markers of iron status or erythropoiesis in cord blood were assessed by linear regression and mediation analysis.

Results: Cord ERFE was detectable in all newborns delivered between 30 - 42 weeks of gestation, and mean concentration at birth was 0.73 ng/mL [95% CI: 0.63, 0.85 ng/mL]. Cord ERFE was on average 0.25 ng/mL lower in newborns of Black compared to White ancestry ($p = 0.04$). Cord ERFE was significantly associated with transferrin receptor ($\beta = 1.17$ $p < 0.001$), ferritin ($\beta = -0.27$ $p < 0.01$), and hemoglobin ($\beta = 0.04$ $p < 0.05$). However, cord hepcidin and the hepcidin/erythropoietin ratio captured the most variance in newborn iron and hematologic status (> 25% variance explained).

Conclusion: Neonates born to teens and women carrying multiples were able to produce ERFE in response to neonatal cord iron status and erythropoietic demand.

ERFE, however, did not capture significant variance in newborn iron or hemoglobin concentrations. In these newborns, cord hepcidin and the hepcidin/erythropoietin ratio explained the most variance in iron status indicators at birth.

Introduction

Anemia and inadequate iron accretion before birth are associated with adverse neonatal outcomes (1, 2). Iron provided to the developing fetus is derived from maternal iron stores and dietary iron absorption, and is dependent on efficient placental transfer of iron (3, 4). To date three hormones are involved in iron regulation and erythropoiesis: erythropoietin (EPO), erythroferrone (ERFE) and hepcidin. Both EPO and hepcidin are produced by the human fetus (5-8), but at present little information is available on ERFE status in newborns. Data are needed to characterize ERFE concentrations at birth and evaluate interactions between ERFE, other fetally produced hormones, and their association with newborn iron status biomarkers.

Erythropoiesis requires iron to support hemoglobin production, therefore physiological regulatory mechanisms are necessary to ensure iron availability for this demand. Erythropoietin (EPO) is a small glycoprotein responsible for regulating erythrocyte production (9) and is produced by the human fetus as early as 16 weeks of gestation (6) beginning in the liver and transitioning to the kidney by late gestation (10, 11). Fetal production of EPO is thought to remain relatively stable during normoxic conditions (12), but is upregulated in response to conditions leading to neonatal hypoxia (8, 13-16). EPO may also have non-erythropoietic roles as it has been linked to tissue protection (8, 17, 18), immune regulation (19), and is potentially regulated by retinoic acid and thyroid hormone in an oxygen dependent manner (20, 21). Hepcidin is the major systemic iron regulatory hormone (22) and is produced by the human fetus as early as the first trimester of pregnancy (5). Hepcidin production by the fetus is positively associated with inflammatory markers and elevated during

infection (23-27). Studies in healthy newborns have found that umbilical cord blood hepcidin concentrations are strongly associated with neonatal iron status at birth (23, 25-32) and are more affected by intrauterine iron status at birth than maternal hepcidin when analyzed using a multiple birth model (29). It does not appear that maternal EPO or hepcidin cross the placenta (33-36) and therefore concentrations of these hormones in umbilical cord blood are assumed to be fetally produced.

In 2014, the hormone ERFE was identified and found to decrease hepcidin expression to increase the availability of circulating iron for erythropoiesis (37). Although a validated human ERFE assay was developed in 2017 (38), few normative data on umbilical cord ERFE concentrations in either preterm or term newborns have been published (39). Animal models have demonstrated that ERFE is produced specifically by the erythroblasts in response to elevated EPO (37). In humans ERFE is induced by elevated EPO in adults with clinical conditions (40, 41) or following rhEPO administration (42). Increases in EPO have been associated with decreased hepcidin (42, 43), leading to increases in iron absorption and iron release from stores (44). Whether ERFE plays a mediating role between EPO and hepcidin, influences placental iron transport or if ERFE crosses the placenta in humans remains uncharacterized.

Due to lack of normative data of iron status indicators in cord blood, and the fact that relationships between some of these indicators differs from that reported in older populations (27), normative neonatal data on iron biomarkers and regulatory hormones are needed. The objective of this paper was to characterize umbilical cord ERFE concentrations in both term and preterm newborns in relation to other neonatal

and maternal iron regulatory hormones and iron status indicators in a large cohort of newborns born to women at greater risk of gestational iron deficiency (ID). A secondary objective was to evaluate the ability of ERFE, hepcidin, EPO, and ratios between these hormones, to explain variance in newborn iron status at birth.

Methods

Participants:

Erythroferrone was measured in umbilical cord blood serum obtained from 164 neonates born to pregnant teens (age < 19 years) receiving prenatal care in Rochester, NY between 2006 – 2012 and 127 neonates (twins, triplets and quadruplets) born to adult women recruited from Strong Memorial Hospital and Highland Hospital in Rochester, NY from 2011 - 2014. (**Figure 7.1**). All studies were approved by the institutional review boards of the University of Rochester and Cornell University and informed consent (or assent and parental consent in individuals < 15 years) was obtained from parents of the newborns studied. Neonatal (27, 29, 35, 45, 46) and maternal (47-51) data have been published.

Gestational age at birth was determined based on self-reported menstrual history and sonogram data or from the known date of in vitro fertilization (IVF) when applicable. If menstrual history and sonogram data differed by > 10 days, the earliest available ultrasound data was used to determine gestational age. Deliveries were classified as preterm (< 37 weeks), early term (37 - 38 weeks) and term (≥ 38 weeks of gestation), with the earliest birth at 30 weeks. Low birth weight (LBW) was defined as birth weight < 2500 g and small for gestational age (SGA) was defined as < 10th percentile for growth rate using Fenton growth charts (52). Neonatal birth data were

extracted from medical charts and maternal demographic information and pre-pregnancy body mass index (ppBMI) was self-reported upon entry into the study.

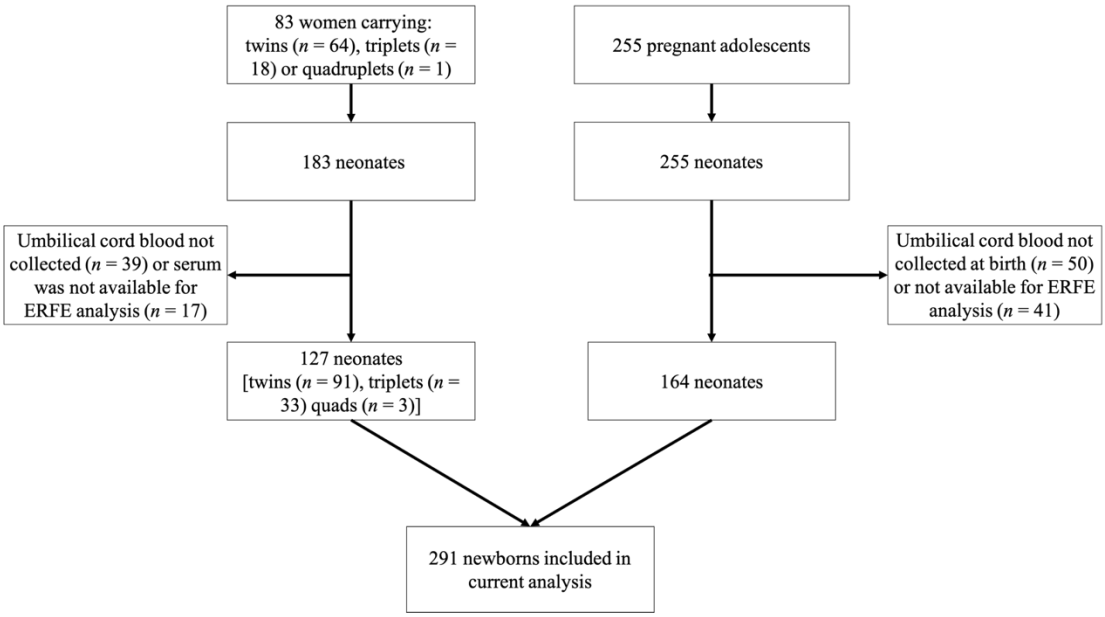

Figure 7.1: Participant Flow Chart

Serum collection and biochemical analysis:

Umbilical cord blood (~15 mL) was collected at birth and nonfasted maternal blood (15 mL) was collected from women at mid-gestation (~26 weeks) and delivery (~39 weeks). Whole blood was sent to the University of Rochester core laboratory for assessment of hemoglobin (Hb) using a Cell-Dyn 4000 hematology analyzer (Abbott diagnostics, Santa Clara, CA). Neonatal anemia was defined as cord Hb < 13.0 g/dL (53). Umbilical cord Hb cutoffs are not typically race adjusted, but since the CDC recommends race adjusting Hb concentrations by 0.4 g/dL in those < 5 y of age (53) we also adjusted for race by decreasing the anemia cut-off for Black newborns to Hb < 12.6 g/dL (53). Neonatal data were evaluated with and without this race-adjustment factor.

Serum ERFE was measured by ELISA (Intrinsic Lifesciences, La Jolla, CA). Maternal and neonatal samples were run on the same plate and the inter-assay CV was 11.4%. 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. Absolute values of this hormone were utilized for statistical analyses. Methods used to analyze the other iron status biomarkers have been published in detail (29, 35). In brief: serum ferritin (SF)(ELISA, RAMCO Laboratories, Stafford, TX), soluble transferrin receptor (sTfR)(ELISA, RAMCO Laboratories, Stafford, TX), serum iron (AAS, Perkin Elmer AAnalyst 800, Waltham, MA), and serum EPO, folate and vitamin B-12 (all Siemens Immulite 2000, Erlangen, Germany) were measured in maternal and neonatal serum. In adults low SF reflects low body iron (ie. iron deficiency)(54) and elevated sTfR reflect increased erythrocyte iron demand (ie. functional iron deficiency)(55). It is unknown if these biomarkers provide the same information in a newborn that prioritizes iron for Hb production at the expense of other tissues (ie. the brain)(56, 57). In the teen cohort serum hepcidin (ELISA, Intrinsic Lifesciences, La Jolla, CA, LOD: 5 ng/mL), serum IL-6 (Millipore Magnetic Multiplex, Temecula CA) and CRP (Siemens Immulite 2000, Erlangen, Germany, LOD: 0.2 mg/L) were measured (35) with different assays than the multiples cohort (hepcidin: ELISA, Bachem, Torrance, CA, LOD 0.39 mg/L; serum IL-6 and CRP: ELISA, R&D Systems, Minneapolis, MN, CRP LOD 0.077 mg/L)(48).

Statistical analysis:

Non-normally distributed values were transformed prior to statistical analysis. Neonatal characteristics and iron status indicators were compared between cohorts by

ANOVA, chi-square or Wilcoxon rank sums test. Total body iron (TBI)(mg/kg) was calculated with the following equation: -[log (sTfR/SF) − 2.8229]/0.1207 (58). Erythrocyte iron (mg/kg) was calculated as previously described (56, 61) assuming the iron content of Hb is 3.47 g/kg (62) and newborn blood volume of 80 mL/kg (63): neonatal Hb (g/L) X blood volume (L/kg) X birth weight (kg) X Hb iron content (mg/g). The storage iron pool (mg/kg) was calculated using the logarithmic relationship between SF and body storage iron (64). The Hep/ERFE and Hep/EPO ratios were calculated to capture both iron status and erythropoietic drive. The ERFE/EPO ratio was expected to better capture erythropoietic drive and hypoxia. Indicators analyzed with different assays between cohorts (CRP, IL-6, and hepcidin) were standardized by dividing each measurement by its respective cohort's mean and dividing this number by the standard deviation of the cohort. All analyses were performed using the multiples cohort maternal identification number as a random coefficient to control for lack of independence in the twins, triplets and quadruplets. Pearson pairwise correlations were assessed between ERFE and iron status, hormones and inflammatory markers. Determinants of ERFE were found through linear mixed models, where neonatal iron status indicators and maternal characteristics with $p < 0.2$ from bivariate correlation analysis were tested simultaneously and eliminated by backward selection until only statistically significant predictors remained. An R2 for linear mixed models was calculated following published methods (65). The intraclass correlation coefficient (ICC) for ERFE was calculated as the between-mother variance divided by the sum of the within- mother and between-mother variances as previously published (29). The above statistical analyses were performed using JMP 14.0 (SAS

Institute Inc). To assess interrelations between ERFE, iron status indicators, Hb and hormones, mediation models were developed using STATA 15 (StataCorp LLC). Finally, interaction terms were evaluated using Johnson-Neyman methods (66) to identify the cut point at which the relationship was no longer significant using R (Rstudio, PBC). All analysis reported were evaluated in the full study cohort unless there was a significant interaction term with the study cohort, in which case separate analyses were presented for comparison purposes.

Results

Neonatal Characteristics:

Newborn characteristics are presented in **Table 7.1**. The majority of neonates born to women carrying multiples were born prematurely (63%) and delivered via cesarean section (72%), while the neonates born to teens tended to be delivered at term (90%) via vaginal delivery (88%). Among the premature multiples neonates, 89% were LBW (n = 77/87) and 21% were SGA (n = 14/66). In the whole population, 49% of newborns were born to Black mothers and 19% of newborns were born to mothers who self-identified as Hispanic. Over 40% of newborns were delivered to mothers who entered pregnancy overweight or obese (based on self-reported ppBMI), and nearly half (49%) of the women gained more than the recommended amount of weight across gestation. Mean neonatal iron status indicators in each cohort are presented in Table 2. Overall, 18% of newborns were anemic at birth, and this value did not significantly change if Hb cutoffs were adjusted for race (18% vs. 17%). The prevalence of anemia was significantly greater prevalence among neonates born to teens ($p = 0.002$). None of the newborns were folate deficient, however one newborn

in the teen cohort was deficient in vitamin B-12. Maternal iron status and further detailed information on neonatal iron homeostasis have been published (29, 35, 46, 48-50).

Neonatal Erythroferrone and Hormone Concentrations

Mean ERFE concentrations, hormone concentrations and ratios between these hormones are presented in **Table 3**. Ratios between these regulatory hormones were explored to capture neonatal adaptive responses in relation to iron status (hepcidin) or to alterations in erythropoietic drive and hypoxia (EPO and ERFE). Umbilical cord ERFE concentrations were measured in 85% of neonates born to women enrolled in the original study cohorts (29, 35, 46) and the CV of duplicate ERFE concentrations was < 10% across the range of values measured. When data on variables from neonates with ERFE data were compared to neonates who did not have sufficient serum for ERFE analysis (n = 50), neonates with ERFE data had lower serum iron ($p = 0.001$) and higher birth weight ($p = 0.03$). There were no other significant differences between the two groups. At birth, mean umbilical cord ERFE concentration averaged 0.73 ng/mL [95% CI: 0.63, 0.85 ng/mL], and neonatal ERFE needed to be log transformed to normalize distribution. Umbilical cord ERFE was significantly lower in neonates who experienced IUGR ($n = 18$, $p = 0.02$). Umbilical cord ERFE did not significantly differ by cohort ($p = 0.71$), maternal ethnicity ($p = 0.61$), maternal parity ($p = 0.39$), maternal ppBMI ($p = 0.40$), sex of the neonate ($p = 0.74$), mode of delivery ($p = 0.74$), LBW classification ($p = 0.98$) or in those born prematurely ($p = 0.66$).

Table 7.1: Characteristics of Newborns

Variable	Whole Population	Multiples Cohort	Teen Cohort
Neonatal Characteristics	**(*n*= 291)**	**(*n*= 127)**	**(*n*= 164)**
Gestational Age, wks	37.7 ± 3.1	34.9 ± 2.4[a]	39.8 ± 1.3*
Early term (37.1 – 38 wks) (%)	50	32	10*
Preterm (32 – 37 wks) (%)	30	60	3*
Very preterm (< 32 wks) (%)	3	8	0*
Birth weight, kg	2.84 ± 0.68	2.29 ± 0.53	3.26 ± 0.43*
LBW (< 2.5 kg) (%)	31	67	3*
VLBW (< 1.5 kg)(%)	2	6	0*
SGA (%)[2]	17	20	15*
LGA (%)[2]	2	0	4*
APGAR (1 min)	7.8 ± 1.5	7.7 ± 1.6	7.8 ± 1.4
Male (%)	47	45	49
Mode of delivery			
Cesarean (%)	38	72	12*
Types of multiples			
Twins (%)	71.7	71.7	-
Triplets (%)	26.0	26.0	-
Quadruplets (%)	2.4	2.4	-

[1]Data presented as mean ± SD or Percentage. *Denotes significant difference (P<0.05) between cohorts. LBW, low birth weight; VLBW, very low birth weight; SGA, small for gestational age.

[2]SGA was defined as < 10th percentile for growth rate using the Fenton growth charts and LGA was defined as > 10th percentile for growth rate using the Fenton growth charts

[3]Gestational weight gain categories were determined using the IOM categories with adjustment for gestational age at delivery. For the teen cohort recommended weight gain was 12.7 – 18.1kg for underweight women, 11.3 15.9kg for normal weight women, 6.8 – 11.3 kg for overweight women gain is 5 – 9.1 kg (11–20 lb) for all obese women. For the multiples cohort, recommended gestational weight gain of 22.7 - 28.1kg 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

Table 7.2: Iron Status Indicators in Umbilical Cord Blood

Variable	*n*	Whole Population	*n*	Multiples Cohort	*n*	Teen Cohort
Hb, g/dL	224	14.8 ± 2.5	112	15.4 ± 2.1*	112	14.2 ± 2.7
Anemia (%)	40	18	11	10[a]	29	26[b]
SF, µg/L	288	111 [102 – 121]	126	104 [90.4 – 119]	162	117 [105 – 132]
Erythrocyte iron[2], (mg/kg)	224	40.9 ± 6.9	112	42.6 ± 5.6*	112	39.2 ± 7.6
Storage iron[3], (mg/kg)	288	15.9 ± 7.5	126	15.3 ± 7.6	162	16.5 ± 7.3
sTfR, mg/L	288	6.8 [6.4 – 7.1]	126	6.0 [5.6 - 6.4]*	162	7.5 [7.1 – 8.0]
sTfR Index[4]	288	3.4 [3.2 – 3.5]	126	3.0 [2.7 – 3.3]*	162	3.7 [3.4 – 3.9]
TBI[5], mg/kg	223	53.6 ± 7.2	112	56.1 ± 6.2*	111	51.0 ± 7.3
Serum Fe, mg/L	253	2.5 [2.3 – 2.7]	124	2.8 [2.6 – 3.1]*	129	2.2 [2.0 – 2.4]
IL-6, pg/mL[6]	-	-	123	3.6 [3.1 – 4.2]	145	9.0 [7.0 – 11.6]
CRP, mg/L[6]	-	-	122	0.10 [0.09 – 0.11]	94	0.28 [0.23 – 0.35]
Undetectable[7] (%)	-	-	39	32	19	20
Folate, nmol/L	201	46.6 [43.7 – 49.7]	117	36.4 [34.3 – 38.7]	84	65.4 [59.9 – 71.5]
< 6.8 nmol/L (%)	0	0	0	0	0	0
Vitamin B_{12}, pmol/L	179	536 [495 – 583]	108	517 [464 – 575]	71	570 [501 – 647]
< 148 pmol/L (%)	1	1	0	0	1	1

[1]Data presented as mean ± SD, Geometric mean [95% CI] for transformed variables, or percentage. *Denotes significant difference ($p < 0.05$) between cohorts. CRP, C-reactive protein; Fe, iron; Hb, hemoglobin; IL-6, interleukin-6; SF, serum ferritin; sTfR, soluble transferrin receptor; TBI, total body iron. [2]Erythrocyte iron (mg/kg) was calculated as neonatal hemoglobin (g/L) X estimated blood volume (L/kg) X birth weight (kg) X 3.4 (mg/g). [3]Storage iron (mg/kg) was calculated using the logarithmic relationship between SF and body storage iron (64). [4]sTfR index was calculated as the following: sTfR/Log_{10}(SF) [5]TBI was calculated with the following equation: TBI (mg/kg) = -[log (sTfR/SF) − 2.8229]/0.1207 [6]IL-6 and CRP were not assessed between cohorts as they were measured with different assays. [7]Undetectable was classified as below the limit of detection for CPR this kit. In the teen cohort this was 0.2 mg/L and in the multiples cohort this was the limit of detection for CRP was 0.077 mg/L

Table 7.3: Umbilical Cord Iron Regulatory Hormone and Hormone Ratio Concentrations

Variable	*n*	Whole Population	*n*	Multiples Cohort	*n*	Teen Cohort
ERFE, ng/mL	291	0.73 [0.63 – 0.85	127	0.77 [0.62 – 0.94]	164	0.71 [0.57 – 0.88]
EPO, mIU/mL	273	24.15 [21.81 – 26.73]	124	17.4 [15.1 – 20.0]	150	31.7 [27.8 – 36.1]*
Hepcidin[2], ng/mL		-	126	13.6 [11.4 – 16.4]	161	96.4 [84.8 – 109.7]
Std hep	287	-0.008 ± 0.99	126	0.04 ± 0.4	161	0.08 ± 0.3
Hep/EPO[2]		-	124	0.8 [0.6 – 1.0]	149	3.1 [2.6 – 3.9]
Std hep/EPO	272	-0.59 ± 14.39	126	-0.002 ± 0.08	149	0.007 ± 0.08
Hep/ERFE[2]		-	126	18.0 [13.2 – 24.5]	161	136 [105 – 177]
Std hep/ERFE	287	0.003 ± 0.08	126	0.2 ± 2.4	161	-1.2 ± 19.1
ERFE/EPO	273	0.03 [0.03 – 0.04]	124	0.04 [0.04 – 0.05]	150	0.02 [0.02 – 0.03]*

[1] Data presented as geometric mean [95% CI]. [*]Denotes significant difference ($p < 0.05$) between cohorts. EPO, erythropoietin; ERFE, erythroferrone Hep, hepcidin; [2]Differences between cohorts were not assessed as hepcidin values were measured with different assays.

Erythroferrone was detectable in all umbilical cord samples obtained from these cohorts, which ranged between 30 – 42 weeks of gestation at birth (Figure 1). All newborns in the multiples cohort were born between 30 - 38 weeks of gestation, and ERFE concentrations did not significantly vary as a function of gestational age at birth (β: 0.01, p = 0.72)(Figure 1A). However, newborns born to teens were generally born between 36 – 42 weeks of gestation and ERFE concentrations significantly increased as a function of gestational age at birth (β: 0.09, p = 0.02) (Figure 1B). After controlling for neonatal EPO or sTfR (two indicators that were also significantly positively associated with GA at birth (35)), the relationship between ERFE and gestational age was no longer significant (both p = 0.5).

Neonatal Erythroferrone Associations with Neonatal Iron Status Indicators and Hormones.

Significant bivariate correlations between umbilical cord ERFE and umbilical cord iron status indicators and hormones are presented in **Table 7.4**. In the whole population, ERFE was significantly positively associated with Hb, sTfR, sTfR index, EPO, and erythrocyte iron and significantly inversely associated with SF and hepcidin. Although newborn ERFE was significantly positively associated with Hb, ERFE concentrations were not significantly lower in anemic neonates (0.61 ng/mL [95% CI: 0.35, 0.78 ng/mL] n = 40) than non-anemic neonates (0.76 ng/mL [95% CI: 0.62, 0.92 ng/mL] n =184)(p = 0.10). The strongest determinants of neonatal ERFE were neonatal EPO and sTfR, which together explained 21% of the variance in ERFE at birth (n = 274) (**Table 7.5**).

Table 7.4: Correlations Between Umbilical Cord Erythroferrone and Iron Status Indicators

	Whole Population	Multiples Cohort	Teen Cohort
Hemoglobin	0.04* (224)	0.09** (112)	0.03 (112)
Serum Ferritin	-0.27** (288)	-0.46*** (126)	-0.01 (162)
Erythrocyte Iron[2]	2.61** (224)	4.42*** (112)	1.11 (112)
Storage Iron[3]	-1.46 (288)	-4.48** (126)	-0.08 (162)
Transferrin Receptor	1.17*** (288)	0.90** (126)	1.47*** (162)
Total Body Iron[4]	0.01 (219)	0.02* (111)	0.00 (108)
sTfR Index[5]	0.94*** (288)	0.79*** (126)	1.15*** (162)
Serum Fe	0.12 (253)	0.06 (124)	0.23 (128)
Erythropoietin	0.76*** (269)	0.72*** (122)	0.73*** (147)
Hepcidin	-0.43*** (283)	-0.48*** (125)	-0.21 (158)

[1]Data presented as correlation coefficients (*n*) from regression models where multiples from one mother was controlled for by random effect. Models were also controlled for maternal race and cohort. *Denotes $p < 0.05$, **Denotes $p < 0.01$, ***Denotes $p < 0.001$. Fe, iron; sTfR, soluble transferrin receptor

[2] Erythrocyte iron (mg/kg) was calculated as neonatal hemoglobin (g/L) X estimated blood volume (L/kg) X birth weight (kg) X 3.4 (mg/g).

[3] Storage iron (mg/kg) was calculated using the logarithmic relationship between SF and body storage iron (64).

[4]Total body iron was calculated with the following equation: TBI (mg/kg) = -[log (sTfR/SF) − 2.8229]/0.1207.

[5]sTfR index was calculated as the following: $sTfR/Log_{10}(SF)$

Table 7.5: Neonatal Determinants of Erythroferrone In Cord Blood

	Whole Population		Multiples Cohort		Teen Cohort	
	β	*p*	β	*p*	β	*p*
EPO	0.58	< 0.001	0.61	< 0.001	0.45	0.002
sTfR	0.66	< 0.001	-0.60	0.03	1.17	< 0.001
Hepcidin			-0.37	0.003		
Erythrocyte iron			0.02	0.01		
Variance explained	21%		45%		25%	
	n = 274		*n* = 110		*n* = 150	

Data presented as correlation coefficients from regression models controlling for race and cohort and the maternal identification number was a random coefficient to account for multiples newborns being born from the same mother.

In all neonates, the positive relationship between ERFE and EPO was modified by neonatal Hb concentration (p-interaction = 0.02). In neonates with cord Hb < 17.7 g/dL, a significant relationship between EPO and ERFE was evident, but in newborns with Hb > 17.7 g/dL (n = 17) no significant relationship between EPO and ERFE was evident (**Figure 7.2**). Newborns with Hb > 17.7g/dL had significantly higher EPO (p = 0.04), ERFE (p = 0.02) and sTfR (p = 0.01), and significantly lower hepcidin (p = 0.02) and SF (p = 0.004). Newborns with Hb > 17.7 g/dL were also on average 103 g smaller at birth (p = 0.03) and more were born SGA (p = 0.01), although no significant differences in the prevalence of preterm birth (p = 0.15) or IUGR (p = 0.35) were evident between groups.

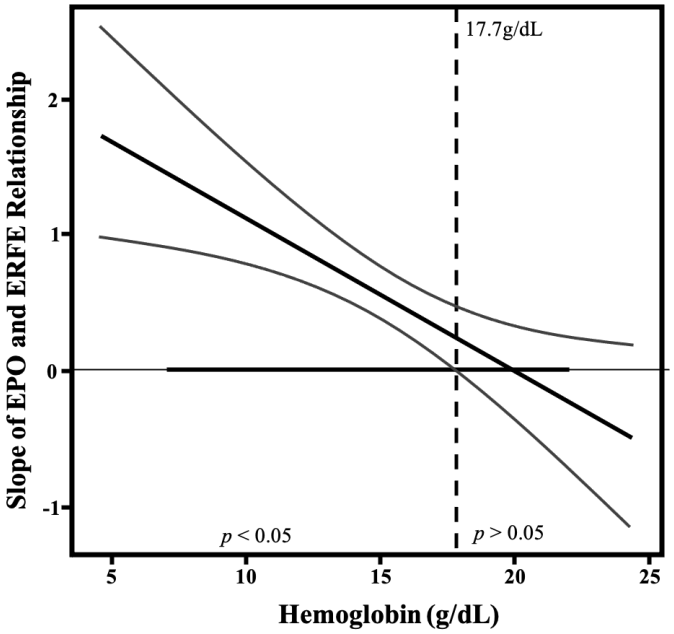

Figure 7.2 Relationship Between Erythropoietin and Erythroferrone in Cord Blood Were Influenced by Cord Blood Hemoglobin Concentrations. The strength and significance of the relationship between EPO and ERFE changes as neonatal hemoglobin concentration increases. The thick solid black diagonal line demonstrates the slope of EPO and ERFE per Hb concentration, with the curved lines being the 95%CI. The thick solid black line on the X axis indicates the range of observed data. The dotted line indicates the hemoglobin concentration (17.7 g/dL) for which neonates with hemoglobin concentrations above this value no longer have a significant relationship between EPO and ERFE, and neonates with hemoglobin concentrations less than 17.7 g/dL have a significant relationship between EPO and ERFE (p < 0.05).

The utility of umbilical cord SF concentrations at birth is not fully understood. Although cord SF has been found to be positively associated with hepcidin, it is also often inversely associated with neonatal Hb concentrations (27). Further evaluation of the relationship between ERFE and SF was undertaken using mediation analysis, and results suggests that the observed relationship between ERFE and SF was indirect with hepcidin as the intermediary (p = 0.003) (**Figure 7.3**). Mediation analysis was also utilized to determine if ERFE played a mediating role between the observed EPO and hepcidin relationship, given that this has been reported in animal models (37). Using this approach, the inverse correlation between EPO and hepcidin was found to only be minimally mediated by ERFE (β: -0.06, p = 0.045) (**Figure 7.3**).

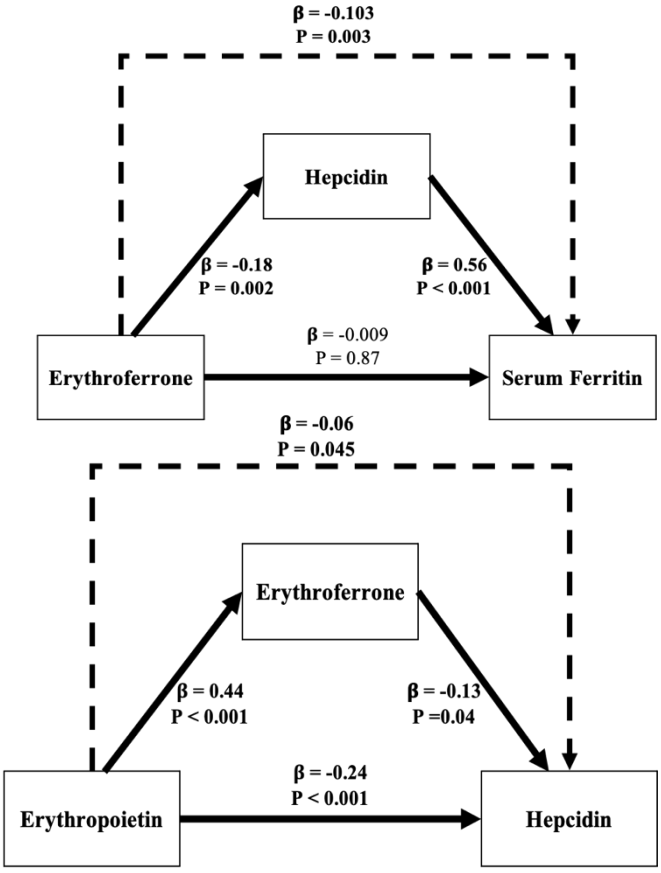

Figure 7.3: Direct and Indirect Relationships Between Iron Regulatory Hormones and Serum Ferritin in Umbilical Cord Blood. Mediation models were utilized to assess direct (consistent lines) and indirect (dashed line) associations between biomarkers in umbilical cord blood. Bolded coefficients and p-values indicate significant effects (p < 0.05).

Ratios between regulatory hormones may provide greater predictive ability in identifying newborns at increased risk of ID or anemia. In the whole population, SF was significantly positively associated with the hep/EPO (β: 0.27, $p < 0.001$, $n = 273$) and hep/ERFE (β: 0.17, $p < 0.001$, $n = 288$) ratios. sTfR was significantly inversely related to the hep/EPO ratio (β: -1.61, $p < 0.001$, $n = 272$) and the hep/ERFE ratio (β: -1.94, $p < 0.001$, $n = 287$) as was the sTfR/log(SF) index [hep/EPO: β: -1.71, $p < 0.001$, $n = 272$; hep/ERFE: β: -1.90, $p < 0.001$, $n = 287$]. An inverse relationship between Hb and the hep/EPO ratio (β: -0.04, $p = 0.02$, $n = 212$) and hep/ERFE ratio (β: -0.08, $p < 0.001$, $n = 222$) was also observed. Although there was a significant inverse association between Hb and the hep/EPO and hep/ERFE ratios, these ratios did not significantly differ between anemic and non-anemic newborns ($p = 0.81$ and $p = 0.09$ respectively). The ERFE/EPO ratio however was significantly higher in anemic compared to non-anemic newborns ($p = 0.03$, $n = 213$). Serum Fe was not significantly associated with any hormone ratio (all $p > 0.1$).

Significant differences in determinants of ERFE were evident between cohorts. A significant positive relationship between ERFE and Hb was found among multiples newborns, and in these newborns ERFE was significantly inversely associated with hepcidin. However, in the newborns born to teens, ERFE was not significantly associated with either Hb or hepcidin. Significant differences in the mode of delivery were evident between cohorts, with a higher prevalence of vaginal delivery in the neonates born to teens. Because hepcidin is known to be significantly associated with the duration of labor (35), these associations were reexamined after controlling for the duration of labor, mode of delivery and neonatal inflammation (IL-6), however this

did not alter the significance of the relationship. Significant differences in the relationship between Hb and the hep/ERFE ratio or the hep/EPO ratio were also evident between cohorts with significant associations between Hb and these two ratios only being evident among the multiple birth newborns ($p < 0.01$).

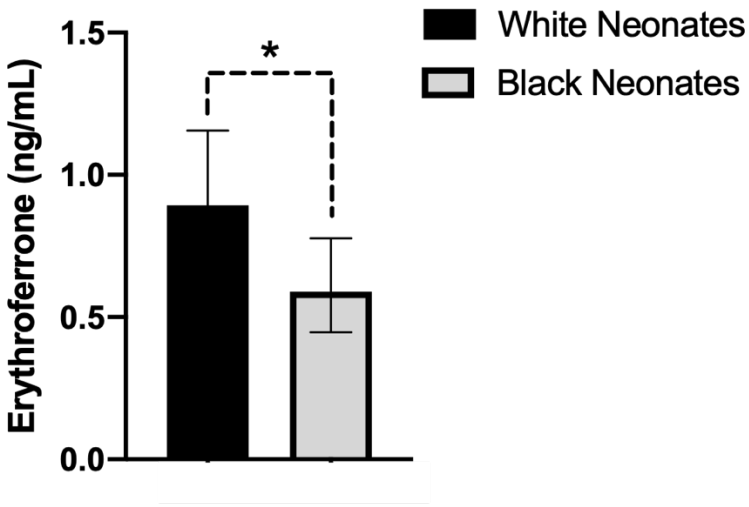

Figure 7.4 Cord Erythroferrone in Neonates of Black or White Women Carrying Multiples and Black or White Teens Carrying Singletons.
Values are geometric mean [95% CI], n = 148 (Black) or n = 141(White). *P < 0.05.

Racial Differences in Neonatal ERFE

Neonatal ERFE was significantly lower in Black compared to White newborns (0.59 ng/mL [95% CI: 0.45, 0.78 ng/mL] vs. 0.89 ng/mL [95% CI: 0.69, 1.16 ng/mL] $p = 0.04$) (**Figure 7.4**), and this difference remained significant after controlling for neonatal Hb ($p = 0.004$) or maternal Hb ($p = 0.003$). The ratio between ERFE/EPO was also significantly lower in Black neonates ($p = 0.02$). The relationship between Hb and ERFE significantly differed as a function of race, whereby a positive association between Hb and ERFE was found in White newborns (β: 0.05, $p = 0.006$, $n = 116$) but no significant association between these indicators was observed in Black

newborns (β: 0.04, p = 0.10, n = 108). Likewise, Hb was only significantly associated with EPO in White newborns (β: 0.03, p = 0.03, n = 110 vs β: 0.01, p = 0.68, n = 103). These differences did not appear to be driven by differences in birth weight (p = 0.2) gestational age at birth (p = 0.81), maternal ppBMI (p = 0.6), maternal BMI at delivery (p = 0.7) or maternal weight gain (p = 0.6).

Neonatal Erythroferrone Associations with Maternal Iron Status Indicators and Hormones

In the whole population, neonatal ERFE was significantly positively associated with maternal ERFE concentrations at mid-gestation (β: 0.43, p < 0.001, n = 199) and at delivery (β: 0.39, p < 0.001, n = 237). Neonatal ERFE was, on average, 26% higher than maternal ERFE concentrations at mid-gestation and 19% higher than maternal ERFE concentrations at delivery. Neonatal ERFE was only significantly positively associated with maternal ERFE/EPO (β: 0.41, p < 0.001, n = 231). Additionally, maternal ERFE (mid-gestation or delivery) was not significantly associated with neonatal iron status or hormone concentrations other than hep/ERFE and ERFE/EPO (both p < 0.001). Neonatal Hb and SF were not associated with any maternal hormone ratios. Neonatal hepcidin concentrations were significantly positively associated with maternal hep/EPO at delivery (β: 0.10, p = 0.003, n = 229) and neonatal EPO was associated with maternal hep/ERFE (β: -0.23, p < 0.001, n = 231). Other relationships between maternal and neonatal iron status indicators have been published (29, 35).

Erythroferrone in Multiple Birth Newborns

Potential differences in ERFE concentrations between the twin, triplet and quadruplet siblings were explored. Mean difference in ERFE between siblings was

1.12 ng/mL and mean differences were significantly greater between the triplets/quad siblings than between twins ($p = 0.03$). Mean differences in ERFE also increased as chorionicity increased ($p = 0.01$) (mono: 0.31 ± 0.55 ng/mL, n = 12; di: 0.92 ± 0.33 ng/mL, n = 33; tri: 2.76 ± 0.61 ng/mL, n = 10). To further evaluate the impact of a shared uterine environment on neonatal ERFE concentrations, ICC values were calculated to assess differences in ERFE concentrations between siblings compared to differences observed between unrelated newborns. Values close to 0.5 suggest equal intra- and inter-uterine variance. The ICC value for ERFE was 0.58 (95%CI: 0.35, 0.64), which was similar to that previously reported for IL-6 and sTfR (29). Controlling for differences in amnionicity and chorionicity did not significantly change the calculated ICC value.

The ability of iron regulatory hormones, and ratios between these hormones, to explain variability in newborn iron status indicators was explored (**Table 7.6**). Cord ERFE explained 31% of variance in cord hepcidin and 45% of variance in cord EPO. However, ERFE was unable to explain a large portion of variance in Hb, sTfR, SF, storage iron or erythrocyte iron. Neonatal hepcidin was able to explain the most variance in cord Hb, erythrocyte iron and the ratio between Hb/SF. Neonatal hepcidin explained more variance in neonatal Hb and iron status than maternal hepcidin. Additionally, the ratio between hep/EPO was able to explain the most variance in sTfR, SF, sTfR index and storage iron.

Table 7.6: Ability of Iron Regulatory Hormones to Explain Variance in Newborn Iron Status Indicators

	Percent Intrauterine Variance Explained By:						
	Cord ERFE	**Cord EPO**	**Cord Hepcidin**	**Maternal Hepcidin**	**Cord H/ERFE**	**Cord H/EPO**	**Cord ERFE/EPO**
Cord Hb	3.7	7.7	25.9	0.6	18.3	22.2	6.2
Cord SF	14.8	29.4	41.8	0.2	36.6	49.1	6.4
Erythrocyte iron[1]	4.5	7.0	26.9	11.5	19.4	22.4	6.7
Storage iron[2]	15.0	29.3	44.7	2.5	37.2	49.2	6.5
sTfR	25.4	40.1	44.0	2.5	47.4	52.8	4.8
Hb/SF	14.7	24.9	48.1	0.1	38.5	18.6	0.9
sTfR Index[3]	25.7	44.7	49.9	0.4	54.0	61.9	4.0
TBI[4]	4.0	8.8	1.0	3.1	1.0	9.3	6.1
Serum Fe	0.9	3.5	0.6	0.6	0.1	4.5	4.3

[1]Intrauterine variance estimates were calculated to determine the amount of variance of each iron status indicator between siblings. Two models were generated; model 1 controlled for neonatal birth weight and model 2 controlled for neonatal birth weight and iron regulatory hormone of choice. The percentage of intrauterine variance explained by the iron regulatory hormone was calculated as variance estimates of (model 1− model 2)/model 1 × 100.

Discussion

To our knowledge, the current study is the first to characterize neonatal ERFE at birth in two populations of newborns of which 18% were anemic at birth. Erythroferrone was detected in all umbilical cord blood samples obtained in newborns born from 30 - 42 weeks of gestation, indicating the developing fetus produces this hormone as early as 30 weeks of gestation. Neonatal ERFE concentrations were higher than maternal ERFE concentrations and were significantly positively related to maternal ERFE. Although these newborns appeared to independently regulate ERFE production in relation to their individual iron status, this newly identified hormone did not capture more variability in neonatal iron or hematologic status than previously identified neonatal regulatory hormones or iron biomarkers. Of all indicators measured, neonatal hepcidin and the ratio between hep/EPO, were most strongly associated with neonatal iron and Hb concentrations.

Neonatal umbilical cord serum ERFE concentration in these newborns averaged 0.73 ng/mL [95%CI: 0.63 – 0.85 ng/mL]. The mean concentration observed in these neonates was 26% higher than maternal ERFE concentrations at mid-gestation and was only 19% higher than maternal values obtained at delivery. Currently, only one publication has reported ERFE concentrations in cord blood within a small cohort (n = 36) of newborns, where healthy term newborn ERFE concentrations were similar to that reported in these cohorts (1.0 ± 0.8 ng/mL, n = 10), and higher values were reported in newborns born to obese or diabetic mothers (2.8 ± 4.1 ng/mL, n = 13)(39). Furthermore, the mean neonatal ERFE concentration was more than 50% higher than published values reported in non-pregnant women (67) or healthy males (42, 67) using

the same assay. The higher ERFE concentration in neonates may be a consequence of adaptation to the hypoxic intrauterine environment.

Erythroferrone is an erythropoietic stress hormone that is produced by erythroblasts in response to stimulation by EPO (37). During fetal development, dynamic changes in erythropoiesis occur as the site of erythroblast production changes (68). The stage of development also impacts the hemoglobin isoform produced. Fetal hemoglobin (HbF) has an increased affinity for oxygen (69) but it is gradually replaced by adult hemoglobin (HbA) shortly after birth. The human fetus begins to produce both hepcidin (5) and EPO (6) relatively early in gestation, but similar data on ERFE are not available. In this cohort of newborns, ERFE was detected in samples obtained in newborns that were born across 30 – 42 weeks of gestation. Additional data are needed to identify the earliest stage at which the human fetus begins to produce this regulatory hormone, but significant associations found between ERFE and iron status biomarkers at birth suggest this hormone supports fetal erythropoietic iron demand in utero.

Under hypoxic conditions, EPO production is increased and binds to EPO-receptors on erythroblasts initiating the JAK/STAT signaling cascade to increase ERFE expression (9, 37). Thus serum ERFE concentrations are a product of circulating EPO and the number of erythroblasts. In these newborns the relationship between EPO and ERFE was influenced by neonatal Hb concentrations but only among newborns with Hb < 17.7 g/dL. A significant relationship was not evident among newborns with cord Hb concentrations > 17.7 g/dL. These newborns were disproportionately SGA with significantly lower SF and higher concentrations of both

EPO and ERFE. We speculate that neonates with Hb concentrations > 17.7 g/dL may have experienced hypoxia as this would explain higher EPO and ERFE stimulating Hb production, and the increased iron utilization for erythropoiesis could lead to a reduction in iron stores.

Erythroferrone is thought to downregulate hepcidin by sequestering BMP2/6 thus limiting the production of hepcidin (70). In these neonates, as expected, ERFE expression was increased in response to EPO, and both EPO and ERFE were inversely associated with hepcidin. We did not however find that ERFE was the main driver of decreased hepcidin concentrations upon EPO stimulation. This finding suggests other hepcidin signaling pathways, such as those that respond to low serum iron, may have a larger regulatory role in this cohort of newborns.

Ratios between iron regulatory hormones may provide increased ability in explaining variability in neonatal iron status than individual hormone measures as ratios may address coordinated regulatory responses to both hypoxia and ID. For example, low hep/ERFE or hep/EPO suggest coexistence of increased erythropoietic activity and ID, whereas, high ERFE/EPO indicates response of erythron to EPO. Using this approach, the hep/EPO explained the most variability in neonatal sTfR, SF and storage iron concentrations. Neonatal hepcidin and hep/EPO explain the most variance of neonatal Hb and erythrocyte iron, which reflect the largest iron pool for the newborn. This suggests that these hormones play a large role in regulating neonatal red blood cell production and thus Hb status. We recently reported similar findings in the mothers of these neonates, whereby ratios between hep/EPO also best predicted maternal iron status (71). In these newborns, the hep/EPO ratio was

inversely associated with Hb and erythrocyte iron and positively associated with SF and storage iron concentrations. This finding is consistent with the significant inverse relationship between SF and Hb previously published in these newborns demonstrating hypoxia driving iron utilization for Hb production (27).

During fetal development adequate Hb production is essential for tissue oxygenation (57), and the fetus prioritizes iron use for RBC production over tissue stores (56, 57). The mass of the erythrocyte iron pool (mg/kg) in these newborns was significantly higher than their storage iron pool (mg/kg). We predicted that a higher prevalence of anemia would be found among multiple birth neonates because these neonates tended to be born prematurely to women who exhibited an increased prevalence of ID and anemia compared to that observed among teen mothers (48). However, we observed that the neonates born to pregnant teens, nearly all of whom were born at term, had a greater prevalence of anemia at birth. This finding may be a consequence of differential nutrient partitioning in teen pregnancies whereby biological immature gravida may prioritize nutrients in support of their own continued growth as has been reported in animal models of adolescent pregnancy (72). Further study of maternal-fetal nutrient partitioning and normative data in teen pregnancies are needed to identify newborns at risk of anemia so interventions can be initiated.

The CDC has set a lower Hb cutoff to define anemia in Black adults and children compared to White individuals (53), due to a shift in Hb distribution between these two groups (73, 74). However, these race specific criteria are frequently not applied, particularly among pediatric populations. In this cohort, prevalence of neonatal anemia did not significantly differ as a function of race, yet neonatal ERFE

concentrations were significantly lower in Black neonates. This relationship remained significant after controlling for either maternal or neonatal Hb concentrations. The ratio between ERFE/EPO was also significantly lower in Black neonates, suggesting that in these neonates more EPO was needed per unit increase in ERFE concentration. There are few other normative data on cord iron status biomarkers in these two groups with which to compare these findings, but given the growing awareness of the impact of genetics on nutrient metabolism (75, 76), more data are needed to understand the mechanisms responsible for ancestry driven differences in iron metabolism.

There are several limitations that impact interpretation of these data. We did not obtain any data on hemoglobin isoform concentrations in these newborns. At the time of birth 55-65% of hemoglobin is HbF (77), however it has been found that infants with severe anemia have elevated fetal Hb at birth because the switch to HbA is delayed (78). We are not aware of other data that have examined if the HbF to HbA transition differs between Black and White newborns.

The wide range of Hb concentrations evident in this group of healthy neonates provided opportunities to characterize determinants of ERFE concentrations in umbilical cord blood and examine how this newly identified hormone was regulated in response to changes in hepcidin, EPO and iron status. Erythroferrone was present in all newborns studied and was produced as early as 30 weeks of gestation. Concentrations of ERFE in umbilical cord serum were significantly inversely related to neonatal iron status indicators and significantly positively related to erythropoietic drive as expected. Umbilical cord ERFE and its associations with erythropoietic indicators significantly differed between Black and White newborns and further data

are needed to explore possible mechanisms responsible for these differences. Using all currently identified hormones in the EPO, ERFE, and hepcidin signaling pathway, low cord blood hep/EPO ratios were most strongly associated with decreased SF and elevated sTfR in newborns. These findings may provide an additional measure of iron status at birth and help identify newborns that may have experienced in utero iron deficiency.

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CHAPTER 8

SUMMARY AND CONCLUSIONS

Summary

This doctoral research describes novel findings on serum ERFE concentrations across pregnancy, ERFE mRNA concentrations within the placenta and serum ERFE concentrations in the newborn. In addition, this research further characterized changes in iron status biomarkers and regulatory hormones across pregnancy and in the newborn at birth, while highlighting potential new biomarkers for identifying women and newborns at increased risk of iron deficiency. This research also shed light on changes in maternal iron absorption across pregnancy, how previous estimates neglect to account for iron transferred to the fetal/placental unit and how different sources of maternal iron are transferred to the neonatal compartment. The three aims of this dissertation project are described in depth in Chapters two through seven. Major findings from each aim are summarized below followed by implications and future directions.

The main findings from aim one have improved our understanding of iron homeostasis and partitioning of different sources of iron between the maternal and neonatal compartment. This aim was accomplished by two means: 1. Measuring iron regulatory hormones and assessing relationships between these hormones and maternal iron/hematological status and 2. Use of stable iron isotopes to trace iron utilization by the mother and her developing fetus. For the first time we characterized ERFE across pregnancy and found that it was significantly associated with maternal Hb and iron status (1). Although we hypothesized that ERFE would be a strong indicator to identify women with increased iron needs, EPO and the ratio between hepcidin/EPO were best able to identify women at risk for ID or anemia. We also

reported for the first time that when measuring iron absorption using only maternal RBC iron incorporation and neglecting iron rapidly transferred to the placental and neonatal compartment, this resulted in a significant underestimation of maternal iron absorption. Furthermore, the degree of underestimation was dependent on maternal iron status (2). We also reported in a longitudinal study that iron absorption increases as gestation progresses, and was significantly associated with decrease in maternal body iron across pregnancy. This is one of the few studies to address this with stable iron isotopes and a sensitive analytical technique. The last study to do this was in 2001 using a less precise mass spectrometer (3). Additionally, this is the first report of RBC turnover in pregnancy, and the lifespan was calculated to be ~95 days, and women with greater iron need (as determined assessing change in total body iron across gestation) had a shorter lifespan (greater red cell turnover). These women with shorter RBC lifespan also transferred more iron from RBC catabolism to their neonate than women with longer RBC half-life, suggesting that iron from the RBC mass serves as a significant source of iron for the fetus when maternal stores are low.

The main findings of the second aim help increase our understanding of placental functioning that differs between adult women and pregnant teens. This was accomplished by measuring hormonal transcripts of ERFE and EPO in placental tissue collected from the pregnant teens and women carrying multiple fetuses. For the first time, we demonstrated that the placenta expresses ERFE and EPO as early as 25 weeks of gestation. Determinants of placental hormone transcript expression differed by cohort. In the multiples cohort, placental ERFE and EPO expression was positively associated with placental iron content and inversely associated with maternal Hb and

body iron stores. However, in the teens, placental ERFE and EPO were positively associated with maternal Hb and iron stores. These findings suggest significant differences in placental functioning in relation to maternal iron status in adult women and teens carrying single fetuses.

The main findings from aim three helped improve our understanding on interpretation of iron status biomarkers in umbilical cord blood, and highlight these relationships are not the same as observed in adults. We highlighted an unexpected inverse association between cord Hb and SF, although >100 studies assumed the relationship was positive as seen in adults (4). Additionally we characterized neonatal ERFE in umbilical cord blood, and found this hormone was significantly positively associated with EPO, a relationship that change depending on neonatal hemoglobin concentration (5). Previously only one study was published in a small cohort (n = 36) of term newborns. As our data was the first to measure ERFE in pre-term and term fetuses, we were able to demonstrate for the first time that the fetus produces ERFE as early as 35 weeks of gestation in response to its Hb concentration and erythropoietic drive.

Limitations and Considerations

This research makes a large contribution on our understanding of maternal placental and neonatal iron homeostasis, however there are several limitations that need to be mentioned. Data used in these analyses were obtained from healthy pregnant individuals, however 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.

In our adolescent and multiples cohort, maternal and neonatal hepcidin was measured using two different assays between the cohorts, making comparisons and interpretation of the hormone ratio difficult to interpret. Within chapter three, the partitioning data only included 5 women carrying multiple fetuses, larger studies using stable iron isotopes in women carrying multiples can help confirm the ability of the fetus to regulate its own iron status. Within chapter four, we assessed changes in RBC enrichment across pregnancy. We assumed a constant blood volume, however we know that there are dynamic changes in plasma volume and RBC mass. Additionally, with our methodology, we are unable to measure how much iron was rapidly transferred to the neonatal compartment vs. was incorporated into maternal RBC and released after catabolism. Furthermore, within chapter 5 we measured placental mRNA concentration of iron regulatory hormones and have confirmed that the placenta expresses these hormones, however we do not know if these are regulated translationally, which may result in stronger associations with maternal or neonatal iron status. Finally, care needs to be taken when assuming that umbilical cord iron status indicators are the same as in adult populations or the same over the first few months of life. We have reported associations between iron status indicators, regulatory hormones and hematologic indices in umbilical cord blood, but dynamic changes happen after birth and association between these indicators in relation to longer term outcomes are needed.

Future Directions

These data have provided insight into maternal, placental and neonatal iron regulatory hormones relationships with each other and other iron status and

hematological indicators. Based on these results, further studies are needed to investigate EPO's ability in identifying women with increased iron need. Additionally, there are clear differences observed between women carrying multiples and pregnant adolescents. These differences should further be elucidated to understand if these are driven by biological immaturity or number of fetuses present. Additionally, this study measured iron regulatory hormones in placental tissue in relation to maternal and neonatal iron status, however molecular mechanisms of placental iron transport still need to be clarified. Lastly, changes in these iron status biomarkers and regulatory hormones over the first few months of life need to be further elucidated, and whether these indicators in cord blood are associated with a functional outcome will further strengthen their utility as an indicator of increased iron need across gestation.

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Appendix:

Table 1: Literature search results

First Author, Year (Ref)	Sample Size, Study Site	Study Description	GA (wk)	Mean Cord Hemoglobin (g/dL)	Mean Cord Ferritin (µg/L)	Ferritin Assay	Hb-SF?
Rios, 1975 (76)	26, USA	Maternal ferritin 1. ≤ 9 ug/L 2. > 9 ug/L	41 ± 2	1. 15.5 ± 1.3[2] 2. 16.1 ± 1.5[2]	1. 100.5 ± 30[2] 2. 117 ± 50[2]	IRMA	No
Hussain, 1977 (77)	52, UK	Iron status in mom and newborn in a normal pregnancy	Term	17 (14.6-21.3)[3]	183.2 (62-313)[3]	IRMA	No
Van Eijk, 1978 (78)	30, Netherlands	Mothers receiving 1. supplements 2. no supplements	Not Reported	1.15.63 ± 1.29[2] 2. 15.63 ± 1.61[2]	1. 174.5 ± 45[2] 2. 168 ± 44[2]	Solid phase RIA	No
Bratlid, 1980 (79)	49, Norway	Iron status in mom and newborn in a normal pregnancy	Not Reported	15.4 ± 1.6[2]	144.4 ± 73.2[2]	RIA	No
MacPhail, 1980 (31)	**103, South America**	**Iron status in mom and newborn in a normal pregnancy**	**Term**	**15.4 ± 1.7[2]**	**71.1 ± 100[2]**	**RIA**	**Yes Inverse**
Puolakka, 1980 (80)	47, Finland	Iron status in mom and newborn in a normal pregnancy	39.7 ± 3.1	15.1 ± 12.0[2]	262 ± 200[2]	Nephelometric technique	No
Celada, 1982 (81)	64, Spain	Iron status in mom and newborn born > 2500g	Full term	15.77 ± 1.61[2]	81.00 ± 49.07[2]	RIA	No
Blot, 1982 (82)	100, France 21	Iron status in mom and newborn in a normal pregnancy	39.8 ± 2.2	16.0 (11.7 – 20.7)[3]	72.5 (10 – 286)[3]	RIA	No

Agrawal, 1983 (83)	51, India	Maternal Hb 1. ≤ 8.6 g/dL 2. 8.7 -11.0 g/dL 3. > 11.0 g/dL	37.7-39.4	1. 15.6 ± 18.10[2] 2. 16.3 ± 19.06[2] 3. 15.6 ± 19.90[2]	1. 29.7 ± 10.93[2] 2. 51.1 ± 19.50[2] 3. 86.2 ± 15.60[2]	RIA	No
Kaul, 1983 (84)	300, USA	Iron, lead and erythrocyte protoporphyin in cord blood	Not Reported	15 ± 1[2]	165 ± 107[2]	RIA	No
Wallenburg, 1984 (85)	44, Amsterdam	Maternal iron supplementation on newborn iron status	> 36	16.4 ± 1.61[2]	208 ± 90[2]	Solid phase RIA	No
Iyer, 1986 (86)	175, India	Iron status in mom and newborn in a normal pregnancy	Term	14.83 ± 2.18[2]	54.9 ± 1.38[2]	ELISA	No
Thavaraj, 1986 (87)	71, India	Iron status in mom and newborn in a normal pregnancy	Pre-term to term	15.3 ± 0.2[2]	93.9 ± 10.3[2]	Ferritin kit Travenol Inc,	No
Milman, 1987 (88)	47, Denmark	Iron status in mom and newborn in a normal pregnancy	Not Reported	16.1 (143-1832)[1]	128 (64-215)[1]	IRMA	No
Milman, 1988 (89)	78, Denmark	ZPP and lead status in mom and newborn in a normal pregnancy	Term	16.0 (140-181)[1]	Mean Not Reported	IRMA	No
Ajayi, 1988 (39)	65, Nigeria	Iron status by parity 1. 1 2. 2 - 4 3. > 4	37-42	1. 17.3 (15.1 – 20.0) 2. 17.3 (13.7 – 21.0) 3. 16.8 (14.4 – 19.9)	1. 152.1 (83.6 – 276.7)[3] 2. 143.1 (82.2 – 248.9)[3] 3. 115.8 (68.5 – 195.8)[3]	ELISA	Yes None
Morton, 1988 (90)	81, London	Iron status at birth and through first year of life	38-41	1. 16.2 ± 0.9[2] 2. 16.0 ±0.8[2]	1. 69 (26-182)[3] 2. 58 (24-146)[3]	RIA	No

Bhargava, 1989 (91)	308, India	Newborn iron status by maternal Hb 1. ≤ 6.0 g/dL 2. 6.1 – 8.0 3. 8.1 – 10.0 4. >10.0	Not Reported	1. 19.1 ± 2.4[2] 2. 15.3 ± 3.7[2] 3. 15.1 ± 2.4[2] 4. 15.6 ± 2.4[2]	1. 101.6 ± 1.5[2] 2. 66.0 ± 1.9[2] 52.5 ± 1.4[2] 4. 56.2 ± 1.4[2]	Micro-ELISA	No
Bhargava, 1991 (92)	308, India	Newborn Iron status in relation to maternal ferritin 1. ≤ 10 ug/L 2. > 10 ug/L	37	1. 15.45 ± 2.66[2] 2. 15.44 ± 2.49[2]	1. 51.21 ± 1.50[2] 2. 56.23 ± 1.38[2]	Micro-ELISA	No
Knight, 1991 (93)	88, USA	Nutrient status of mom and neonatal outcomes	Term	14 ± 0.5[2]	74.5 ± 4.9[2]	RIA	No
Lao, 1991 (32)	**96, China**	**Iron status in mom and baby in a normal pregnancy**	**Not Reported**	**15.6 ± 1.9[2]**	**120.39 ± 0.73[2]**	**Double antibody RIA**	**Yes Inverse**
Carpani, 1992 (37)	47, Italy	Cord blood iron status from percutaneous umbilical sampling	18 - 35	Mean not reported	Mean not reported	Not reported	Yes Positive
Hawa, 1993 (40)	201, Saudi Arabia	Iron status in mom and baby in a normal pregnancy	Not reported	15.6 ± 1.6[2]	122.6 ± 82.6[2]	RIA	Yes None
Gaspar, 1993 (94)	157, Mexico	Iron status in mom and baby in a normal pregnancy	>32	16.7 ± 1.3[2]	122.6 ± 6.7[2]	ELISA	No
Altinkaynak, 1994 (95)	52, Turkey	Iron status in mom and baby in a normal pregnancy	38 – 40	16.0 ± 1.5[2]	130.0 ± 54.1[2]	IRMA	No
Barton, 1994 (96)	97, Ireland	Maternal iron supplementation on baby iron status	40	1. 16.71 ± 0.22[2] 2. 17.30 ± 0.64[2]	1. 156.34 ±13.27[2] 2. 165.23± 14.24[2]	RIA	No

Kavukcu, 1994 (97)	27, Turkey	Iron status in mom and baby in a normal pregnancy	Not reported	1. 16.6 ± 6.8[2] 2. 16.1 ±.8.2[2]	1. 75.1 ± 11.0[2] 2. 81.2 ± 16.3[2]	RIA	No
Knight, 1994 (98)	349, USA	Iron status in mother and baby during pregnancy	Not reported	12.9 ± 0.4[2]	69.3 ± 4.5[2]	RIA	No
Hokama, 1996 (99)	53, Japan	Maternal 1. Hb ≤ 11.0 g/dL SF ≤ 7.1 ug/L 2. Hb > 11.0 g/dL SF ≤ 7.1 ug/L 3. > 11.0 g/dL > 7.1 ug/L	Term	1. 16.2 ± 0.4[1] 2. 15.8 ± 0.3[1] 3. 16.4 ± 0.7[1]	1. 77.1 ± 30[1] 2. 81.5 ± 20[1] 3. 90.1 ± 15[1]	ELISA	No
Carpani, 1996 (100)	20, Italy	Newborn erythropoiesis	20-40	Mean not reported	Mean not reported	ELISA	No
Rusia, 1996 (101)	81, India	Transferrin receptor in newborns to evaluate iron deficiency	Term	1. 16.1 ± 1.8[2] 2. 15.7 ± 1.3[2]	1. 112.1 ± 10.2[2] 2. 118.7 ± 120.2[2]	EIA	No
Singla, 1996 (102)	76, India	Maternal Hb 1. ≤ 6.0 g/dL 2. 6.1 – 8.5 g/dL 3. 8.6 – 10.9 g/dL 4. > 11.0 g/dL	37-41	1 14.0 ± 12[2] 2 16.2 ± 25[2] 3 16.4 ± 19[2] 4 17.6 ± 14[2]	1 29.20 ± 11.77[2] 2 57.66 ± 28.06[2] 3.107.88 ± 53.36[2] 4 98.86 ± 32.93[2]	ELISA	No
Preziosi, 1997 (103)	198, Niger	1. Placebo 2. Iron supplement	Not Reported	1. 13.3 ± 2.0[2] 2. 13.6 ± 2.2[2]	1. 97.51 ± 51[2] 2. 107 ± 56[2]	ELISA	No
Kilbride, 1999 (35)	**59, Jordan**	**Maternal anemia and newborn iron status 1. Anemic 2. Control**	**> 37**	**1. 15.2 ± 1.5[2] 1. 15.2 ± 1.5[2]**	**1. 195 ± 149[2] 2. 200 ± 124[2]**	**ELISA**	**Yes Inverse**
Rondo, 1999 (104)	356, Brazil	Maternal iron status and newborn 1. IUGR 2. AGA	Term	1. 16.63 ± 1.96[2] 2. 15.70 ± 1.65[2]	1. 129.66 ± 85.35[2] 2. 133.08 ± 70.78[2]	IA	No

Choi, 2000 (105)	572, Korea	Newborn iron status by newborn sex 1. Males 2. Females	38 ± 3	1. 15.9 ± 1.0[2] 2. 15.9 ± 1.0[2]	1. 182.65 ± 55.20[2] 2. 183.89 ± 53.70[2]	RIA	No
Zavaleta, 2000 (106)	545, Peru	Maternal supplements 1. Iron folate zinc 2. Iron folate	37-38	1. 17.5 ± 23[2] 2. 17.7 ± 21[2]	1. 167.3 ± 75[2] 2. 167.0 ± 100[2]	ELISA	No
Harthoorn-Lasthuizen, 2001 (41)	103, Amsterdam	Maternal iron deficient erythropoiesis in relation to newborn iron status	36-42	16.3 (116-197)[3]	102 (11-395)[3]	ELISA	Yes None
Sweet, 2001 (107)	67, Ireland	Mother 1. Iron replete 2. Iron depleted 3. Smoker 4. Non-smoker	37-42	1. 16.8 (156-176)[3] 2. 15.6 (144-168)[3] 3. 16.8 (159-175)[3] 4. 15.7 (138-171)[3]	1. 171 (121-259)[3] 2. 113 (71-191)[3] 3. 123 (80-171)[3] 4. 190 (113-262)[3]	RIA	No
Sweet, 2001 (108)	144, Ireland	Newborn 1. Very preterm 2. Preterm 3. Term	1. 24-29 2. 30-36 3. 37-41	1. 16.3 (1.6)[1] 2. 15.8 (2.1)[1] 3. 16.1 (1.7)[1]	1. 75 (44-117)[4] 2. 90 (45-142)[4] 3. 131 (90-238)[4]	RIA	No
Erdem, 2002 (42)	44, Turkey	Maternal Hb 1. ≤ 10.0g/dL 2. > 10.0g/dL	Term	1. 16.11 ± 0.39[2] 2. 16.57 ± 1.35[2]	1. 176.9 ± 18.9 2. 251.1 ± 80.6	CIA	Yes None
Gupta, 2002 (109)	58, India	Newborn iron Status by randomized grouping 1. Early cord clamping 2. Delayed cord clamping	39 ± 1.2	1. 13.9 ± 1.5[2] 2. 14.1 ± 1.4[2]	1. 148.1 (42-400)[3] 2. 124.9 (3-615)[3]	EI	No
O'Brien, 2003 (110)	41, Peru	Maternal supplementation 1. Iron 2. Zinc and Iron 3. Control	30 - 36	1. 15.5 ± 15[2] 2. 16.3 ± 14[2] 3. 15.0 ± 16[2]	1. 201.5 ± 107.6[2] 2. 187.5 ± 66.9[2] 3. 170.1 ± 70.7[2]	ELISA	No

Aygun, 2004 (44)	32, Turkey	Newborn iron status in 1. Rhesus hemolytic disease (RHD) 2. Control	35 ± 2	1. 10.7 ± 3.2[2] 2. Not reported	1. 404.9 ± 229[2] 2. 148.6 ± 118[2]	Not Reported	Yes None in RHD group
Emery, 2004 (111)	124, New Zealand	Mothers 1. Maori 2. Non-Maori	> 36	1. 14.36 2. 14.41	1. 83.6 2. 119.8	Abbott IMX	No
Abrams, 2005 (112)	90, Malawi	Neonates born to women who were 1. Infected 2. Uninfected	Not Reported	1. 15.9 (14.5 – 16.9)[3] 2. 15.6 (14.5 – 17.1)[3]	1. 135.5 (66.7 – 267.3)[3] 2. 99 (39.4 – 158.7)[3]	ELISA	No
Lott, 2005 (113)	39, USA	1. Control 2. IUGR 3. FDM	24 - 42	Erythrocyte Hb 1. 34.7 2. 35.6 3. 34	1. 187 2. 147 3. 71	RIA	No
Milman, 2005 (43)	122, Denmark	Maternal iron supplementation on newborn iron status	>39	15.79 ± 1.61[2]	131.83 ± 1.97[2]	EIA	Yes None
Jaime-Perez, 2005 (114)	201, Mexico	Iron status in mom and newborn in a normal pregnancy	Term	15.6 ± 10[2]	81.0 ± 63[2]	RIA	No
Wachs, 2005 (115)	**148, Peru**	**Newborn iron status and temperament**	**32-39 2 < 37**	**15.6 ± 1.4[2]**	**152.19 ± 78.67[2]**	**Not reported**	**Yes Inverse**
Ervasti, 2007 (45)	199, Finland	Newborn iron and hematological status relationships	37- 42	15.9 ± 15[2]	198 ± 137[2]	IEMA	Yes None
Kleven, 2007 (116)	49, USA	1. AGA 2. LGA	39 ± 1.2	1. 15.7 ± 1.0[2] 2. 16.04 ± 0.9[2]	1. 216 ± 69[2] 2. 205 ± 86[2]	ELISA	No

Paiva, 2007 (117)	95, Brazil	Iron status in mom and newborn in a normal pregnancy	37-42	10 - 20[5]	50 - 200[5]	CLIA	No
Pearson, 2007 (118)	3, USA	iron overloaded Beta-Thalassemia mother 1. first pregnancy 2. one twin from second 3. other twin from second	35-36	1. 14.5 2. 19.2 3. 18.5	1. 42 2. 227 3. 203	Not reported	No
Baumann - Blackmore, 2008 (119)	122, USA	1. African Americans 2. Caucasian 3. Hispanic	Term	1. 14.8 ± 1.7[2] 2. 15.4 ± 1.7[2] 3. 14.8 ± 2.5[2]	1. 206.8 ± 129.5[2] 2. 271.9 ± 87.5[2] 3. 250.8 ± 75.4[2]	ELISA	No
Kumar, 2008 (120)	75, India	Maternal Hb 1. ≤ 6.0 g/dL 2. 6.1-8.5 g/dL 3. 8.6 -10.9 g/dL 4. >11.0 g/dL	37–41	1. 15.6 ± 6[2] 2. 15.8 ±11[2] 3. 16.6 ± 8[2] 4. 17.2 ± 7[2]	1. 38.05 ±9.22[2] 2. 56.46 ± 8.41[2] 3. 263 ± 189.39[2] 4. 180.98 ± 164.84[2]	ELISA	No
Ervasti, 2008 (71)	193, Finland	Iron status in cord blood in relation to EPO and pH	> 37	16.0	166	IEMA	No
Mahajan, 2008 (46)	300, India	Maternal Hb 1. > 11.0g/dL 2. > 10 -10.9 g/dL 3. < 6.9 g/dL	Term	1. 12.99 ± 1.15[2] 2. 12.87 ± 1.33[2] 3. 11.42 ± 3.00[2]	1. 97.53 ± 50.6[2] 2. 104.59 ± 53.3[2] 3. 117.24 ± 54.4	Not Reported	No
Ervasti, 2009 (121)	192, Finland	Newborn iron status by maternal iron status 1. %HYPOm < 1% 2. %HYPOm > 1%	>37	1. 16.0 ± 1.5[2] 2. 16.0 ± 1.4[2]	1. 204 ± 145[2] 2. 188 ± 122[2]	IEMA	No
Ali, 2009 (122)	125, Sudan	Newborn iron status by maternal Hb 1. < 11 2. > 11	>37	1. 14.62 ± 1.77[2] 2. 14.49 ± 2.02[2]	1. 245.0 (68.5-679.0)[4] 2. 66.0 (56-231.5)[4]	RIA	No

Imamoglu, 2010 (123)	74, Turkey	Iron status in mother and newborn in pregnancy	> 37	15.00 ± 1.58[2]	163.80 ± 91.98[2]	CLIA	No
Rehu, 2010 (124)	191, Finland	Newborn iron status by Maternal iron stores 1. %HYPOm > 3.4% 2. ferritin < 12 and %HYPOm ≤ 3.4% 3. ferritin > 12 and %HYPOm ≤ 3.4%	40 ± 1.0	1. 15.8 (14.6 - 17.0)[3] 2. 15.7 (15.1 – 16.3)[3] 3. 15.8 (15.4 – 16.3)[3]	1. 134.0 (86.0 – 220.7)[3] 2. 182.2 (142.9 – 233.0)[3] 2. 140.2 (119.0 165.3)[3]	IEMA	No
Young, 2010 (125)	92, USA	Iron status in mother and newborn in adolescent pregnancy	40 ± 1.2	14.7 ± 1.7[2]	114.1 ± 81[2]	EIA	No
Adediran, 2011 (126)	142, Nigeria	Newborn iron status	Not Reported	13.02 ± 2.40[2]	70.85 ± 97.07[2]	ELISA	No
Ozkiraz, 2011 (127)	25, Turkey	Neonates considered 1. AGA 2. SGA	37-40	1. 14.0 ± 1.3[2] 2. 19.3 ± 1.5[2]	1. 385.7 ± 164.8[2] 2. 146.6 ± 91.1[2]	Not Reported	No
El-Farrash, 2012 (128)	90, Egypt	Newborn iron status by maternal Hb 1. > 11 2. 8.6 – 11.0 3. ≤ 8.5	> 37	1. 17.7±1.4[2] 2. 14.4 ± 1.3[2] 3. 11.6 ± 1.1[2]	1. 129.7 ± 45.8[2] 2. 107.1 ± 63[2] 3. 52.8 ± 46.6[2]	ELISA	No
Shao, 2012 (129)	3702, China	Iron status in mother and newborn in a normal pregnancy	37-40	15.4 ± 1.6[2]	189 ± 105[2]	CLIA	No
Mukhopadhyay, 2012 (130)	90, India	Newborn iron status by 1. SGA 2. AGA	35-38	1. 17.1 2. 16.2	1. 68 2. 120	ELISA	No

Young, 2012 (61)	19, USA	Maternal and Newborn utilization of heme and non-heme iron	36 – 41	13.4 ± 30[2]	135 ± 110[2]	ELISA	No
Adediran, 2013 (131)	142, Nigeria	Newborn iron status by maternal Hb 1. ≤ 11.0 2. > 11.0	Not Reported	1.12.54 ± 2.54[2] 2. 13.44 ± 2.23[2]	1. 69.38 ± 78.77[2] 2. 7.26 ± 115.60[2]	ELISA	No
Bolat, 2013 (132)	63, Turkey	Newborn iron status by maternal health status 1. Hypertension 2. Healthy	27-39	1. 16.1 ± 1.4[2] 2. 15.2 ± 1.1[2]	1. 164.7 ± 143.8[2] 2. 198.7 ± 107.4[2]	CLIA	No
Briana, 2013 (133)	151, Greece	Newborn iron status by 1. AGA 2. IUGR	Term	1. 17.5 ± 2.0[2] 2. 20 ± 1.5[2]	1. 92.25 ± 55.62[2] 2. 72.05 ± 47.69[2]	EIA	No
Ozdemir, 2013 (134)	76, Turkey	Newborn irons status by 1. Term 2. Late-preterm	34 - 41.2	16.5 (13.6-21)[3] 17.6 (14-21)[3]	223 (49-565)[3] 242 (46 -767)[3]	CLIA	No
Patidar, 2013 (135)	100, India	Newborn iron status by 1. SGA 2. AGA	1. 38.2 ± 1.4 2. 37.8 ± 0.92	1. 16.4 ± 2.8[2] 2. 15 ± 2.0[2]	1. 103.68 ± 65.4[2] 2. 158.13 ± 98.4[2]	CLIA	No
Cao, 2014 (136)	57, USA	Maternal newborn and placental nutrient regulation	37-41	13.7 ± 2.7[2]	133.0 ± 79.4[2]	ELISA	No
Gupta, 2014 (137)	100, India	Maternal supplementation 1. IV Iron 2. Oral Iron	38.31 ± 1.47	15.8 ± 0.7[2] 15.6 ± 0.7[2]	155.77 ± 46.34[2] 147.68 ± 39.05[2]	ELISA	No
Lorenz, 2014 (63)	223, Germany	Gestational age 1. 24 – 29 weeks 2. 30 – 36 weeks 3. 37 – 42 weeks1	24 - 41	1. 16.8 (15.1 – 18)[4] 2. 16.1 ± 2.2[2] 3. 16.0 ± 2.0[2]	1. 83.0 (44.3 – 119.3)[4] 2. 68 (33.6 – 124.3)[4] 3. 130 (67.8 – 188.3)	IA	No

Morton, 2014 (138)	131, New Zealand	Iron status in mom and newborn in a normal pregnancy	5 < 37 123 > 37	(13.1 – 19.0)[5]	(26 – 287)[5]	CMIA	No
Phillips, 2014 (139)	316, USA	Iron status in obese moms and newborn	>35	16.2 ± 2[2]	149 ± 5[2]	IRMA	No
Basu, 2015 (140)	284, India	Maternal Hb 1. > 11 2. 7 – 11.0 3. < 7	37-39	1. 16.3 ± 1.5[2] 2. 15.8 ± 1.8[2] 3. 12.5 ± 1.0[2]	1. 138.4 ± 32.5[2] 2. 98.7 ± 25.8[2] 3. 40.2 ± 11.7[2]	Immunoturbidimetric	No
De Sa, 2015 (141)	50, Brazil	Iron status in mom and newborn in a normal pregnancy	39.1 ± 1.7	14.1 ± 1.6[2]	122.9 ± 62.4[2]	ELISA	No
Jaime-Pérez, 2015 (142)	187, Mexico	Iron status in mom and newborn in a normal pregnancy	Term	15.69 ± 1.89[2]	79.30 ± 55.14[2]	ELISA	No
Terefe, 2015 (143)	89, Ethiopia	Iron status in mom and newborn in a normal pregnancy	Not reported	16.2 (15.0-17.2)[3]	187.6 (140-264.7)[3]	IA	No
Weigert, 2015 (144)	97, USA	Neonatal eosinophilia 1. low eosinophils < 470 2. high eosinophils > 470	> 38	1. 16.0 ± 0.3[2] 2. 16.3 ± 0.7[2]	1. 146.9 ± 10.6[2] 2. 109.5 ± 28.3[2]	ELISA	No
Wibowo, 2015 (145)	100, Jakarta	Multi-micronutrient and protein supplement during pregnancy	> 36	13.24 ± 1.93[2]	153.45 (40-1050)[4]	Not stated	No
Zhao, 2015 (146)	1,595, China	Maternal supplementation 1. placebo/folate 2. iron/folate	39	1. 15.2 (15.1-15.3)[3] 2. 15.3 (15.2-15.4)[3]	1. 103 (97.9-102.1)[3] 2. 105 (101-110)[3]	CLIA	No

Armony-Sivan, 2016 (147)	80, China	Neonates considered 1. pre and postnatal ID 2. prenatal ID 3. postnatal ID 4. No ID	39.6	1. 13.8 ± 1.8[2] 2. 14.5 ± 1.9[2] 3. 14.3 ± 3.2[2] 4. 14.7 ± 1.7[2]	1. 84.5 ± 27.7[2] 2. 73.5 ± 79.8[2] 3. 202.4 ± 78.0[2] 4. 177.8 ± 58.6[2]	ELISA	No
Basu, 2016 (36)	45, India	Iron deficiency and Maternal Hb 1. > 11.0 2. < 7.0	37-39	1. 16.3 ± 16[2] 2. 12.2 ± 10[2]	1. 159.7 ± 26.3[2] 2. 55.4 ± 19.7[2]	Immunoturbidimetric	Yes positive
Berglund, 2016 (148)	186, Spain	1. Normal weight 2. Overweight or Obese 3. Gestational Diabetes	Not Reported	1. 16.7 ± 1.6[2] 2. 17.0 ± 2.4[2] 3. 16.3 ± 3.7[2]	1. 168 (103 – 265)[4] 2. 163 (115 – 233)[4] 3. 139 (88-268)[4]	Not Stated	No
Cao, 2016 (149)	230, USA	Maternal ppBMI 1. < 18.5 2. 18.5 – 24.9 3. 25.0 – 29.9 4. > 30	39 ± 3	1. 13.4 ± 2.4[2] 2. 14.0 ± 2.8[2] 3. 14.0 ± 2.3[2] 4. 15.5 ± 2.4[2]	1. 198.2 ± 179.0[2] 2. 147.4 ± 94.7[2] 3. 133.3 ± 105.9[2] 4. 144.6 ± 95.5[2]	ELISA	No
Dosch, 2016 (150)	85, USA	Maternal BMI 1. < 35 2. > 35	> 37	1. 14.6 ± 1.4[2] 2. 14.5 ± 1.7[2]	1. 76.4 (55.3 – 128.6)[4] 2. 64.7 (43.0 – 80.3)[4]	ELISA	No
Ertekin, 2016 (151)	150, Turkey	Umbilical cord clamping times 1. 30 seconds 2. 90-120 seconds	> 38	1. 15.4 ± 1.5[2] 2. 15.2 ± 1.8[2]	1. 222.0 ± 200.0[2] 2. 171.9 ± 114.8[2]	Not Reported	No
Lee, 2016 (14)	**193, USA**	**Gestational Age 1. 37 – 39 weeks 2. 39 – 41 weeks 3. > 41 weeks**	**37.5 – 41.4**	**1. 14.6 ± 2.4[2] 2. 14.2 ± 2.8[2] 3. 14.7 ± 2.1[2]**	**1. 122 (91-161)[4] 2. 114 (100-130)[4] 3. 108 (80-144)[4]**	**ELISA**	**Yes Inverse**
Lou, 2016 (152)	115, China	Prenatal iron deficiency and a neurophysiological outcome	Term	15.5 ± 15.09[2]	130.82 ± 80.06[2]	CLIA	No
Mireku, 2016 (153)	636, Africa	Cord serum ferritin concentrations and cognition	N = 27 < 37 N = 487 > 37	14.0 ± 2.3[2]	176 ± 171.56[2]	IA	No

Yang, 2016 (154)	83, China	Mothers with 1. Gestational diabetes 2. No gestational diabetes	36 - 39	1. 15.8 2. 15.5	1. 172.75 2. 111.60	CLIA	No
Basu, 2017 (37)	70, India	Maternal iron deficiency and fetal neural development	37 - 41	1. 14.2 ± 1.8[2] 2. 16.6 ± 1.2[2]	1. 68.51 ± 31.1[2] 2. 161.9 ± 30.0[2]	CLIA	Yes positive
Jobarteh, 2017 (155)	301, Gambia	Maternal newborn and placental nutrient regulation	40 ± 1.5	(13 – 15)[5]	(175 – 330)[5]	IA	No
MacQueen, 2017 (156)	24, USA	1. SGA n =1 2. IDM n=1 3. VLBW n=3 4. preterm n=1	25 - 40	1. 16.3 2. 15.4 3. 16.8 4. 17.9	1. 11 2. 35 3. 28 4. 24	Not Reported	No
Stadem, 2017 (157)	300, Uganda	Neonates considered 1. Glucose-6-phosphate deficient 2. Normal	Not Reported	1. 14.0 2. 14.4	1. 211.6 2. 100.5	ELISA	No
Basu, 2018 (158)	90, India	Neonates born to mothers 1. With iron deficiency anemia 2. Without iron deficiency anemia	37 - 41	1. 14.2 ± 1.8[2] 2. 16.6 ± 1.2[2]	1. 68.5 ± 31.1[2] 2. 161.9 ± 30.0[2]	Clinical Chemistry Auto-Analyzer RXSuzuka	No
Hua, 2018 (159)	751, China	Newborn iron stores by gestational age	39.7 ± 1.0	Not reported	Not reported	CLIA	No
Ru 2018 (16)	**183, USA**	**Iron Status in Multiples 1. Preterm 2. Early-term**	**1. 33.4 ± 0.3 2. 37.5 ± 0.07**	**1. 15.1 ± 0.3[1] 2. 15.3 ± 0.4[1]**	**1. 96.4 [79.7, 116.7][3] 2. 112.8 [85.8, 148.3][3]**	**ELISA**	**Yes**
Ru 2018 (34)	183, USA	Iron Status in Multiples 1. Preterm 2. Early-term	1. 33.4 ± 0.3 2. 37.5 ± 0.07	1. 15.1 ± 0.3 2. 15.3 ± 0.4	1. 96.4 [79.7, 116.7] 2. 112.8 [85.8, 148.3]	ELISA	No

Singhal, 2018 (160)	100, India	Association between newborn and maternal hemoglobin and ferritin	37 - 41	Not Reported	15.89 ± 0.58^{2}	Not Reported	No

Appendix Table 2: Summary of Iron Absorption Studies

Stable Isotope

	Whittaker[1,2] (n = 9)	Barrett[1,2,5] (n = 12)	Whittaker[1,3] (n = 5)	O'Brien[3] (n = 43)	Delaney[3] (n = 5)	Young[3,5] (n = 20)	Current study[1,3] (n = 15)	Koeing (n=50)
Fe tracer dose (mg)	5.01 As FeSO4	2.83 As $FeSO_4$	4.7 As $FeSO_4$	10 As FeSO4	9 As $FeSO_4$	9 As $FeSO_4$	20 As $FeSO_4$	8.4 As FeSO4
Total iron load (mg)	5.23	6	10	60	9	9	20	8.4
Isotope	Fe^{54}	Fe^{54}	Fe^{57}	Fe^{57}	Fe^{57}	Fe^{57}	Fe^{57}	Fe^{57}
Age (y)	NR	NR	22-34	18-35	31±2	20±4	18-34	28±7
Mean Hb (g/dL)	12wk 12.4 24wk 11.6 36wk 11.0	12wk 12.7 24wk 11.6 36wk 11.6	12wk 12.3 36wk 12.0	T3 11.3	11.6	11.2	12.2 11.0 11.6	10.8
Week	**Geometric mean [Range]**	**Geometric mean [Range]**	**Geometric mean [Range]**	**Median [Range]**	**Median [Range]**	**Median [Range]**	**Median [Range]**	**Median [IQR]**
< 10								
10 - 14	7.6 [1.2,21.8]	7.2 [4.9,10.9]	11.8 [4.4,24.8]				8.5 [5.0,27.6]	
15 - 19								
20 - 24	21.1 [8.6,58.4]	36.3 [27.6,47.3]						
25 - 29								
30 - 34				14.0 [0.8,33.2]	37.9 [19.0,62.1]	40.5 [18.3,62.9]		9.8 [0 – 20]
> 35	36.3 [18.4,55.6]	66.1 [57.1,76.2]	49.0 [38.2,77.2]	9.3 [1.3,15.6]			18.8 [6.2,34.2]	
Fold Change	4.78	9.2	4.15				2.22	

	Radioactive Isotope				
	Balfour[3] (n=13)	**Svanberg[1,4] (n = 30)**	**Svanberg[1,4] (n = 30) Iron treated**	**Hahn[3] (n = 12 -56)**	**Svanberg[1,4,5] (n = 22)**
Fe Load with tracer (mg)	1 – 122 As ferric ammonium citrate	100 As $FeSO_4$	100 As $FeSO_4$	2 – 9 As $FeCl_2$	4 As $FeSO_4$
Dose	NR	100nCi	100nCi	200,000-1,000,000 countable counts	100 nCi
Isotope	NR	Fe^{59}	Fe^{59}	Fe^{59}	Fe^{59}
Age (y)	21-42	NR	NR	NR	18-30
Hb (g/dL)	Hematocrit 29-42	12wk 12.5 24wk 11.5 36wk 11.4	12wk 12.5 24wk 11.6 36wk 12.4	NR	12wk 12.4 24wk 11.6 36wk 10.9
Week of Gestation	**Percent uptake [Range]**	**Mean [Range]**	**Mean [Range]**	**Median percent uptake**	**Mean [Range]**
< 10				13.75	
10 - 14	2.7	6.5 [1.2,11]	6.7 [3.4,14.5]	13.75	1.5 [0,6.7]
15 - 19	4.2			21.25	
20 - 24	3.2	9.2 [3.2,17.9]	6.0 [1.1,13.1]	40	5.8 [0,15.4]
25 - 29	2.5			41.25	
30 - 34	16.4			50.625	
> 35	9.1 [2.2,27.7]	14.3 [5.9,24.7]	8.6 [2.7,15.0]	51.25	14.6 [5.7,28.3]
Fold Increase	3.27	2.2	1.28	3.72	9.7

Compared to non-pregnant women (DRI 18% absorption). Hahn estimations were adjusted for 80% incorporated into RBC since 100% incorporation was used in the publication.

[1]Longitudinal study/ measures across gestation

[2]Serum iron measured shortly after dosing to calculate absorption

[3]RBC iron incorporation used to calculate absorption

[4] Whole body counter

[5]Iron isotope dose given with food

Appendix Form 1

Division of Maternal-Fetal Medicine

UNIVERSITY of ROCHESTER MEDICAL CENTER

Eva K. Pressman, MD
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Consent Form

Study Title: Sources of Fetal Iron during Pregnancy
Principal Investigator: Eva Pressman, MD.
Co-Investigator: Kimberly O'Brien, PhD.
Elizabeth Cooper, EdD.
Study Recruiter: Melissa Miller, MPH.
Lauren Cowen, BA.
Sarah Caveglia, BS.

Introduction

This consent form describes a research study and what you may expect if you decide to participate. You are encouraged to read this consent form carefully and to ask the person who presents it any further questions you may have before making your decision whether or not to participate.

This study is being conducted by Eva Pressman, MD, and Kimberly O'Brien, PhD, from the University of Rochester's Department of Obstetrics and Gynecology and Highland Hospital's Department of Obstetrics and Gynecology. Kimberly O'Brien is also on the faculty at Cornell University.

You are being asked to participate in this study because you are pregnant and are between 15-35 years of age.

Purpose of Study

The purpose of the study is to learn more about how pregnant adolescents and adult women supply iron to their developing baby while they are pregnant. We are also interested in looking at how your iron status during pregnancy will affect how much iron your baby has in his or her blood when your baby is born.

Background

Iron is an important nutrient that helps deliver oxygen from our lungs to other body parts. It helps the muscle use and store oxygen. In addition, iron is a part of many compounds that are needed to keep the body healthy during pregnancy. Babies need iron for normal growth and development. Too little iron in the baby may delay the development of many important organs including the brain and may increase the risks of certain diseases when the baby becomes an adult.

During pregnancy, women get their iron from either of the two iron sources: 1) iron in the foods and iron supplements they eat (dietary sources) and 2) the iron stored in their body (storage sources). Scientists do not yet know how these two iron sources are used by pregnant women and how they are used to support the babies' growth during pregnancy. This study will look at how the body uses dietary and maternal storage iron during pregnancy.

In this study, we will give you two different forms of iron orally. These forms of iron are present naturally in your body and in the foods you eat. Iron in our food and in our body has 4 different weights. Some of these forms of iron are very rare. We can give these rare forms of iron to you in a flavored syrup and measure how much of these are then found in your blood and in the cord blood when your baby is born. This allows us to see how you use the iron stored in your body and how much of this maternal storage iron is used to supply iron to your baby during pregnancy.

Description of Study Procedures

If you decide to participate in this study, you will be asked questions to determine if you understand what this study is about. You will be asked some questions to see how healthy you are. We will also look in your medical chart to learn more about your health and to learn more about your pregnancy. We will collect some blood from you during your pregnancy and some cord blood and placental samples when your baby is born. At the time of enrollment, we will:

- Ask you questions about what you currently eat and if you take any prenatal supplements or vitamins/ minerals.

- Ask you questions about how often you use drugs or smoke cigarettes.

Your participation in this study will last from when you are about 14-16 weeks pregnant, and will end after your baby is born.

First part of the study during pregnancy: You will have a total of five study visits during your pregnancy. We will try to schedule the study visits at the same time as your normal prenatal clinic visits (either the Rochester Adolescent Maternity Program (RAMP) or the University of Rochester Medical Center (URMC) Midwifery Group).

The activities included in these 5 study visits are as follows:

- **Visit 1 (study week 1)** will take about 2 hours. When you are about 14-16 weeks pregnant, you will be asked to come to the RAMP clinic or URMC Midwifery Group office in the morning for the first study visit. Your weight will be measured and a blood sample (1 teaspoon) will be taken from your arm. You will be asked to drink a special form of iron (^{57}Fe) (in about 1-2 teaspoons of flavored raspberry syrup). The iron needs to be taken on an empty stomach, so you will be asked to not have breakfast before you come for the appointment. If you do eat breakfast that morning, we will ask you to wait in the office for about 1 ½ hours so that your stomach is empty before you drink the iron dose. After you take the iron, please do not eat or drink anything except distilled water for the next 1 ½ hours. At the end of this study visit, you will be given a packaged lunch that you can choose to eat in the office (we can prepare it for you) or for you to take home and eat. The lunch contains the foods listed below:

    1. A Frozen meal (Healthy Choice, Turkey, Chicken or vegetarian entrée-340 g, 1 serving, pre-packaged container)
    2. A bag of pretzels (30 g, snack size personal pack)
    3. A bottle of water (17oz)

- **Visit 2 (study week 3) and Visit 3 (study week 11)** will last less than 30 min. You will be asked to return to RAMP clinic or URMC

Midwifery Group office for a blood draw (2 teaspoons). Your weight will also be measured at each visit.

- **Visit 4 (study week 19)** will take about 2 hours. When you are about 33-35 weeks pregnant, you will be asked to come to the RAMP clinic or URMC Midwifery Group office in the morning. Your weight will be measured and a blood sample (2 teaspoons) will be taken from you. Same as before, you will be asked to drink a special form of iron (^{58}Fe) (in about 1-2 teaspoons of flavored raspberry syrup). The iron needs to be taken on an empty stomach, so you will be asked to not have breakfast before you come for the appointment. If you do eat breakfast that morning, we will ask you to wait in the office for about 1 ½ hours so that your stomach is empty before you drink the iron dose. After you take the iron, please do not eat or drink anything except distilled water for the next 1 ½ hours. At the end of the study visit, you will be given a packaged lunch that you can choose to eat in the office (we can prepare it for you) or to take home and eat. The lunch will contain the same foods as the lunch offered on visit 1.

- **Visit 5 (study week 21)** will last less than 30 min and we will collect a blood sample (2 teaspoons) from you. We will also measure your weight.

If you decide to participate in this study, your medical chart will have a sticker put on it. The sticker will let the doctors and nurses know that they should call us after you go into labor and before your baby is born.

Second part of the study: delivery samples

When you enter the hospital to have your baby, we will:

- Collect a sample of your blood (2 teaspoons). The extra blood sample will be drawn at the same time as a blood sample is normally taken from your arm when you enter the hospital to have your baby. We will use this sample to measure how much of the two special forms of iron you consumed on visit 1 and visit 4 are present in your blood as well as the proteins and hormones that regulate your iron status.
- Record the birth weight and birth length and other health information on your baby from your baby's medical chart.
- After a baby is born, the baby's afterbirth (called the **placenta)** comes out. The placenta is usually thrown away. Before it is

thrown away we will take some pieces of the placenta. This will help us to learn more about how your placenta sent nutrients to your baby while you were pregnant.

- Take some blood (2 tablespoons) from the cord of the placenta. This cord blood sample will tell us about how much of the two special forms of iron you consumed on Visit 1 and Visit 4 were transferred to the baby before he/she is born. We will also measure some proteins and hormones that regulate the baby's iron status in this cord blood sample and will measure the amount of iron that your baby has at birth using this blood sample.
- We will keep all the blood and tissue samples until the entire sample has been used to measure nutrients.

Number of Subjects

We expect a total of 24 pregnant adolescents (15-18 y) and adult women (19-35 y) will participate in this study.

Duration of Subject Involvement

Your participation in the study will last about 6-7 months.

Potential Risks and Discomforts of Participation

The stable isotopes of iron that you will consume are naturally occurring forms of iron that are already present in small amounts in our diet and in our body. There are no known risks associated with their consumption.

When the blood samples are taken, you may get a bruise and it may hurt a bit. Some people feel lightheaded or faint when their blood is drawn. We will reduce these risks by having only a trained phlebotomist obtain these samples and we will use all standard techniques in this process.

There are no known risks from the collection of the placental samples and cord blood at delivery. The samples of the placenta are collected after the baby is born and will not cause any risk to your baby.

Benefits of Participation

There is no direct benefit to you for participating in this study.

Alternatives to Participation

You do not have to participate in this study if you do not want to. Your decision not to join this study will not affect the health care you receive at RAMP clinic, URMC Midwifery Group office, Highland Hospital or elsewhere.

Payments

You will be paid a total of $160 as gift cards from Walmart for completing the study. The amount of money associated with the gift card you will receive at the completion of each study component is listed below:

- $50 at Visit 1
- $15 at Visit 2
- $15 at Visit 3
- $50 at Visit 4
- $15 at Visit 5
- $15 at Delivery

Confidentiality of Records and HIPAA Authorization

While we will make every effort to keep information we learn about you private, this cannot be guaranteed. Other people may need to see the information. While they normally protect the privacy of the information, they may not be required to do so by law. Results of the research may be presented at meetings or in publications, but your name will not be used.

The federal Health Insurance Portability and Accountability Act (HIPAA) requires us to get your permission to use health information about you that we either create or use as part of the research. This permission is called an Authorization. We will use**:**

- Demographic information (where you live, your phone number, etc.)
- Information on your height, weight and previous pregnancies
- Dietary information and information on supplement use over pregnancy
- Self-reported drug and use of cigarettes
- Current use of medications and prescription drugs
- Diagnosis of any pregnancy complications or health problems
- Test results on hemoglobin and routine tests drawn across pregnancy
- The place where you were seen
- The name of your physician
- The medical records of your newborn

We will use your health information to conduct the study and to determine how your health status and other medical care issues that are happening during your pregnancy might be influencing iron status in you and your baby. Health information is used to report results of research to sponsors and federal regulators. The health information

collected may be audited to make sure we are following regulations, policies and study plans. University of Rochester Medical Center (URMC)/Strong Health policies let you see and copy health information we have gathered for this research study after the study ends, but not until the study is completed. If you have never received a copy of the URMC/ Strong Health HIPAA Notice of Privacy Practices, please ask the investigator for one.

To meet regulations or for reasons related to this research, the study investigator may share a copy of this consent form and records that identify you with the following people: the Department of Health and Human Services; Cornell University; University of Rochester; Highland Hospital; and your primary care provider.
If you decide to take part, your Authorization for this study will not expire unless you cancel (revoke) it. The information collected during your participation will be kept indefinitely. You can always cancel this Authorization by writing to the study investigator. If you cancel your Authorization, you will also be removed from the study. However, standard medical care and any other benefits to which you are otherwise entitled will not be affected. Canceling you Authorization only affects uses and sharing of information after the study investigator gets your written request. Information gathered before then may need to be used and given to others.
As stated in the section on Voluntary Participation below, you can also refuse to sign this consent/Authorization and not be part of the study. You can also tell us you want to leave the study at any time without canceling the Authorization. By signing this consent form, you give us permission to use and/or share your health information as stated above.

Contact Persons for Questions

For more information concerning this research, or if you believe that you have suffered a research-related injury, please contact: Melissa Miller or Lauren Cowen in the RAMP clinic at (585) 410-0119, Sarah Caveglia in the RAMP clinic at (585)410-5381, Eva Pressman at (585) 275-7480, Elizabeth Cooper at (585) 275-7892, or Kimberly O'Brien at (607) 255-3743.

You can also contact your research advocate at the RAMP clinic, Cindy Schutt, RN, if you have any questions or concerns about the study. She can be reached by phone at (585) 275-7892.

If you have any questions about your rights as a research subject, or any concerns or complaints, you may contact the Human Subjects Protection Specialist at the University of Rochester Research Subjects

Review Board, Box 315, 601 Elmwood Avenue, Rochester, NY 14642-8315, Telephone (585) 276-0005, for long-distance you may call toll-free (877) 449-4441. You may also call these numbers if you cannot reach the research staff or wish to talk to someone else.

Voluntary Participation

Participation in this study is voluntary. You are free not to participate or to withdraw at any time, for whatever reason, without risking loss of present or future care you would otherwise expect to receive. In the event that you do withdraw from this study, the information you have already provided will be kept in a confidential manner.

Blood Samples for DNA

If it is okay with you we would also like to collect white blood cells from your blood so that we can screen for DNA and genes involved in how the body uses iron. The samples may be used to help identify genetic factors that influence how the body uses nutrients and to understand how these may be related to differences in iron status. The samples will not be sold or used directly for the production of commercial products and will be kept in a locked lab. Reports about future research done with the sample will NOT be kept in your health records, but the sample reports may be kept with study records or in other secure areas.

You can decide if you want your sample to be used for this type of research. Your decision can be changed at any time by notifying the study doctor in writing. Your decision about your sample will not affect your participation in this study or other studies.

Please check one:

☐ YES, you may use my blood sample for the DNA studies described above.

☐ NO, you may not use my sample for the DNA studies described above.

Signature/Dates

Subject Consent
I have read (or have had read to me) the contents of this consent form and have been encouraged to ask questions. I have received answers to my questions. I agree to participate in this study. I have received a signed copy of this form for my records and future reference.

Subject Name (print): _____

Signature of Subject: _____

Date: _____

Person Obtaining Consent
I have read this form to the subject and/or the parent/guardian has read this form. I will provide the subject and parent/guardian (if present) with a signed copy of this consent form. An explanation of the research was given and questions from the subject were solicited and answered to the subject's satisfaction. I have given the subject adequate opportunity to read the consent before signing. In my judgment, the subject has demonstrated comprehension of the information.

Name and Title (print): _____

Signature of Person Obtaining Consent: _____

Date: _____