IRON ABSORTPION IN PREGNANT AND NON-PREGNANT WOMEN AND MECHANISMS OF PLACENTAL IRON TRANSFER

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IRON ABSORTPION IN PREGNANT AND NON-PREGNANT WOMEN AND MECHANISMS OF PLACENTAL IRON TRANSFER

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Iron deficiency anemia is the most common nutrient deficiency in the world, with highest rates in pregnant women and young children. Despite the magnitude and severity of iron deficiency, there remains gaps in our knowledge of iron metabolism. Hepcidin is a key regulator of iron homeostasis yet limited data is available on its role during pregnancy. Although our knowledge of non-heme iron metabolism has increased substantially in recent years, the mechanisms regulating heme iron metabolism remain elusive. The overall goal of this research is to further understand the primary determinants of iron absorption and placental iron transfer in women of childbearing age.

To address these issues stable iron isotopes (^{57}Fe and ^{58}Fe) were used to measure iron absorption in non-pregnant (ages 18-32 years, n = 30) and pregnant (ages 16-32 years, n = 20) subjects and placental iron transfer in pregnant women. Non-heme iron absorption was inversely associated with iron status and serum hepcidin. However, heme iron absorption was not significantly associated with serum hepcidin or iron stores, suggesting differential regulation. In a subset of 18 non-pregnant subjects serum prohepcidin (hepcidin precursor) was also assessed but was not significantly associated with measures of iron absorption or serum hepcidin. Both pregnant and non-pregnant women absorbed significantly more iron from a heme

based meal compared to a non-heme iron supplement. Interestingly there also appears to be preferential fetal utilization of maternally ingested heme iron compared to non-heme iron during pregnancy. Iron status in the maternal-neonatal dyad and maternal serum hepcidin were inversely associated with heme and non-heme placental iron transfer. In a cohort of 92 pregnant adolescents (ages 14-18 years), the placental protein expression of transferrin receptor was likewise increased in response to altered iron stores in the mother and neonate. Despite increased iron absorption by women during pregnancy, placental iron transporter expression and placental iron transfer, many of the mothers and the neonates had suboptimal iron status at the time of delivery. Based on study results, further research on the mechanisms of heme iron metabolism and mechanisms of placental iron transfer are warranted.

BIOGRAPHICAL SKETCH

Melissa Fox Young was born in Pratt, Kansas on April 6th, 1984 to Lloyd and Jeanne Fox. Melissa graduated from Lyndon High School in 2002 and then went to Iowa State University. After changing her major at least 10 times she graduated in Nutritional Science with honors in 2006. At Iowa State Melissa met her husband and best friend Brad Young. She worked with Dr. Wendy White, Dr. Manju Reddy and Dr. Grace Marquis in the nutrition department and developed an interest in micronutrient deficiencies and malnutrition. Through an internship at the World Food Prize, in Des Moines, Iowa, she also gained a broader prospective on global food issues. Melissa entered the Ph.D. program in Human Nutrition in the Division of Nutritional Sciences at Cornell University in 2006. Melissa worked in Dr. Kimberly O'Brien's research group conducting research on iron deficiency and iron bioavailability in pregnant adolescents and young women.

To my husband ~ my rock ~

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AA	amino acids
AAS	atomic absorption spectrophotometry
BCRP	breast cancer resistance protein
DCYTB	duodenal cytochrome B
DMT1	divalent metal transporter
Fe	iron
FLVCR	feline leukemia virus receptor C
FnR	ferritin receptor
FP-1	ferroportin
Ft	ferritin
Hb	hemoglobin
HCP1	heme carrier protein
HFE	human hemochromatosis protein
HO	heme oxygenase
Hp	hepaestin
IRE	iron response elements
IRP	iron regulatory proteins
IV	intravenous
PCFT	proton coupled folate transporter
PE	preeclampsia
RAMP	Rochester adolescent maternity program
RBC	red blood cell
SF	serum ferritin
STB	syncytiotrophoblast
TBI	total body iron
Tf	transferrin
TfR	transferrin receptor
TIMS	thermal ionization mass spectrometry
UTR	untranslated regions

CHAPTER 1

INTRODUCTION

Specific Aims

Iron deficiency anemia is the most common nutrient deficiency in the world. The highest rates are in pregnant women and young children due to the increased iron requirements. The prevention of iron deficiency during pregnancy is critical to avoid increased risk of labor and delivery complications, infant and maternal mortality, and potential long term adverse cognitive and developmental outcomes for the affected child (1). Iron homeostasis is meticulously regulated in response to iron stores, erythropoiesis and inflammation/infection through a complex system of post-transcriptional regulation and hormonal (hepcidin) mechanisms. Although our knowledge of non-heme iron metabolism has increased substantially in recent years, the mechanisms regulating heme iron metabolism remain elusive. Furthermore, the dynamics of both heme and non-heme placental iron transfer during pregnancy are largely unknown. Accumulating data suggests that the ability to upregulate iron transfer to the fetus may not be sufficient to insure an optimal iron status in the neonate when maternal iron stores are limited.

The goals of this research were fourfold: first, to characterize the relationship of serum hepcidin and prohepcidin to non-heme iron absorption in non-pregnant women; second, to characterize the determinants of heme and non-heme iron absorption in non-pregnant and pregnant women; third, to determine the role of maternal/infant iron status on heme and non-heme iron transport to the neonate; and fourth, to characterize determinants of a key cellular protein involved in placental iron transport. To address these issues stable iron isotopes were used to measure iron absorption in women of childbearing age and placental iron transfer in pregnant women.

The Specific Aims and Hypotheses are:

I. **To assess relationships between serum prohepcidin and hepcidin concentrations with iron absorption and compare the strength of these relationships between a non-heme food based matrix (orange-fleshed sweet potato) and a supplemental source of iron (ferrous sulfate).**

Hypothesis: Iron absorption is more significantly associated with serum hepcidin than prohepcidin and the strength of the relationships observed between regulatory compounds and iron absorption is stronger for supplemental non-heme iron sources than for food based non-heme sources.

II. **To assess the impact of iron status and serum hepcidin on heme and non-heme iron absorption in non-pregnant and pregnant women.**

Hypothesis: Iron absorption from both heme and non-heme iron is inversely related to iron status and serum hepcidin. However, the strength of these relationships will be higher for non-heme iron absorption than for heme iron absorption.

III. **To elucidate the determinants of heme and non-heme placental iron transfer to the neonate over the last trimester of pregnancy.**

Hypothesis: Heme and non-heme placental iron transfer are inversely related to maternal and fetal iron status as well as maternal serum hepcidin.

IV. **To assess the relationships between placental TfR protein expression and maternal and neonatal iron status.**

Hypothesis: Expression of placental TfR protein expression is inversely related with both maternal and neonatal iron status.

The long-term goals of this project are:

1. To provide data that will add to existing literature on the nutritional value of various sources of iron, based on their relative bioavailability. These data will provide additional information that may contribute to data used in the establishment of US dietary guidance on iron intake recommendations.
2. To elucidate mechanisms of maternal / fetal iron partitioning.
3. To provide mechanistic information on proteins integral to placental iron transfer during pregnancy and determinants of their expression.

Background and Significance

Iron (Fe) deficiency anemia is the most common nutrient deficiency in the world, globally affecting over 1.6 billion people (2). The prevalence of iron deficiency is most severe in children and women in developing countries. However, it also prominent in industrialized nations as well and data for the U.S. indicates that approximately 7.8 million adolescent girls and women of childbearing age have iron deficiency and 3.3 million have iron deficiency anemia (3).

The prevalence rates are even more alarming when the severe consequences of iron deficiency are considered. These include: fatigue, impaired cognitive function, reduced work capacity, and decreased immunity (3;4). Anemia during the first two trimesters of pregnancy has been associated with labor and delivery complications, increased risk of preterm delivery, low birth weight, reduced infant iron status, impaired mother-child interactions as well as infant and maternal mortality (5-8). Risks of iron deficiency during pregnancy are especially high because pregnancy associated iron losses approximate 480-1150 mg (including losses to the fetus, placenta and blood loss at delivery) (9). In a 55 kg woman the total iron requirement during pregnancy is approximately 1040 mg and corresponds to daily absorbed iron

needs of roughly 0.8 mg, 4-5 mg, and 6-8 mg/day for each of three trimesters of pregnancy, respectively (10). Many women struggle to meet the iron demands required for non-pregnant women (1.5 mg/day), so meeting these increased requirements during pregnancy from dietary sources alone is especially difficult. Pregnant adolescents are at an even higher risk of developing iron deficiency compared with pregnant adults because they also face the increased nutrient demands of growth and development. During adolescence, girls have a peak weight gain of 9 kg/year, which requires an additional 280 mg of iron to maintain circulating hemoglobin (Hb) concentrations (11). Thus, even otherwise healthy adolescents are at increased risk of developing iron deficiency due to these high iron demands. Over 60% of 1100 pregnant African-American adolescents studied in a previous medical chart review in Baltimore, Maryland were anemic by the third trimester of pregnancy (12).

Previous research has shown that pregnant women can upregulate non-heme iron absorption in response to iron deficiency, although absorption only increases by 1.5% for every 10 ug/L decrease in serum ferritin (measure of iron stores) concentrations (13). A number of studies have also shown increases in non-heme iron absorption across gestation even in iron replete women (14-17) and in non-pregnant women (18). However, to date no human studies to our knowledge have examined heme iron absorption during pregnancy. Despite the demonstrated upregulation of non-heme iron absorption, it may not be sufficient to insure adequate iron status in the neonate and mother. Unlike other minerals, once iron is absorbed, the body has no mechanism for excreting excess iron so the regulation and control of iron absorption at the enterocyte is vitally important. Iron absorption is meticulously regulated in response to iron body stores, erythropoiesis, and inflammation/infection through a complex system of posttranscriptional regulation and hormonal (hepcidin) mechanisms (19). Understanding of the hormonal control of iron absorption is a rapidly evolving;

hepcidin, the primary hormone believed to be responsible for the systemic coordination of iron homeostasis was only recently discovered in 2000 (20;21). Many questions remain regarding the mechanisms of heme and non-heme iron absorption and our knowledge of placental iron transport is extremely limited. Further information is required on these mechanisms so that appropriate recommendations can be made to help insure adequate neonatal iron stores at birth.

I. Enterocyte Iron Metabolism

Over the past decade, there have been immense advances in our knowledge of intestinal non-heme iron absorption and regulation (19). Dietary non-heme iron in the ferrous state (Fe^{+2}) enters the enterocyte from the gut lumen through a divalent metal transporter (DMT1). Iron that reaches the gut in the ferric state can also be transported across the enterocyte but must first be reduced ($Fe^{+3} \rightarrow Fe^{+2}$) by duodenal cytochrome B (DCYTB) on the brush boarder (apical) membrane before it is transported by DMT1. Non-heme iron that enters the enterocyte is either stored as ferritin or exported across the basolateral membrane through ferroportin (FP-1), also previously referred to as iron regulated transporter protein-1. This export process involves hephaestin (Hp), a ferroxidase, which oxidizes ferrous iron to ferric (Fe^{+3}) iron in order for it to be exported across the basolateral membrane via FP-1 and subsequently incorporated into serum transferrin (Tf).

Intestinal uptake of heme Fe differs markedly from that of non-heme Fe. Recently the elusive heme transporter was thought to be discovered (heme carrier protein, HCP1) (22). However, upon further investigation, Qui et al. demonstrated that HCP1 was actually a proton coupled folate transporter (PCFT) and a poor heme transporter at best, which leaves the primary heme transporter in both the intestine and placenta yet to be fully characterized (23). It has also been hypothesized that heme

iron is taken up by receptor mediated endocytosis, however, the mechanisms have not been worked out for this pathway either (24). Furthermore, it is currently unknown if once heme iron enters the intestinal cell if it is transported across the basolateral membrane intact via one of the recently identified heme transporters, BCRP (breast cancer resistance protein, also known as ABCG2) or FLVCR (feline leukaemic virus receptor C) (25;26). Alternatively, heme may be catabolized by heme oxygenase (HO) within the enterocyte so that it can then enter a common inorganic iron pool with non-heme iron (27). **Figure 1.1.a and Figure 1.1.b** illustrate the pathways of iron absorption and the two proposed models of heme iron metabolism in the enterocyte. It is important to note that the true model may involve a combination of the two models. The ability to trace iron's cellular flux through the unique use of stable iron isotopes in this research allows for further insight into heme iron metabolism.

I a. Regulation of Enterocyte Iron Absorption

Iron absorption is primarily influenced by the biochemical form of iron in the gut lumen (heme or non-heme), the iron status of the individual and the bioavailability of the iron source (based on meal composition and the presence of enhancers or inhibitors such as ascorbic acid and phytic acid, respectively) (28). Common non-heme iron sources are staple crops such as rice, wheat, maize, and root crops. Non-heme iron is the form of iron used in most iron supplements. Heme iron on the other hand, is found primarily from meat sources. Unfortunately, meat is often beyond the reach of the poor due to price and availability or religious/cultural beliefs. In many countries non-heme iron provides the primary source of dietary iron. Heme iron is readily absorbed, is less influenced by iron status and is less affected by other dietary factors compared with non-heme iron (28;29). The percentage of heme iron absorbed is roughly 5-10 fold higher than that of non-heme iron (29).

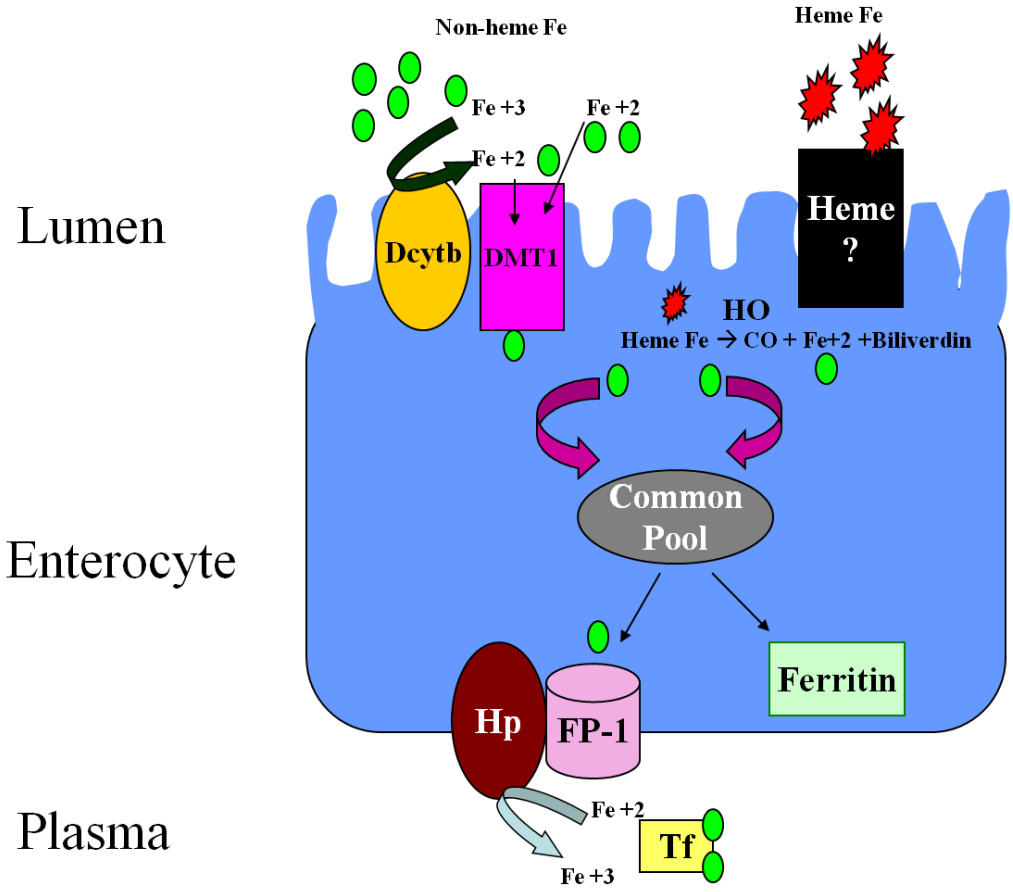

Figure 1.1.a Mechanisms of Iron Absorption I

Heme iron is catabolized by heme oxygenase (HO) and enters an inorganic iron pool with non-heme iron and thereafter joins the same intracellular pathway as non-heme iron.

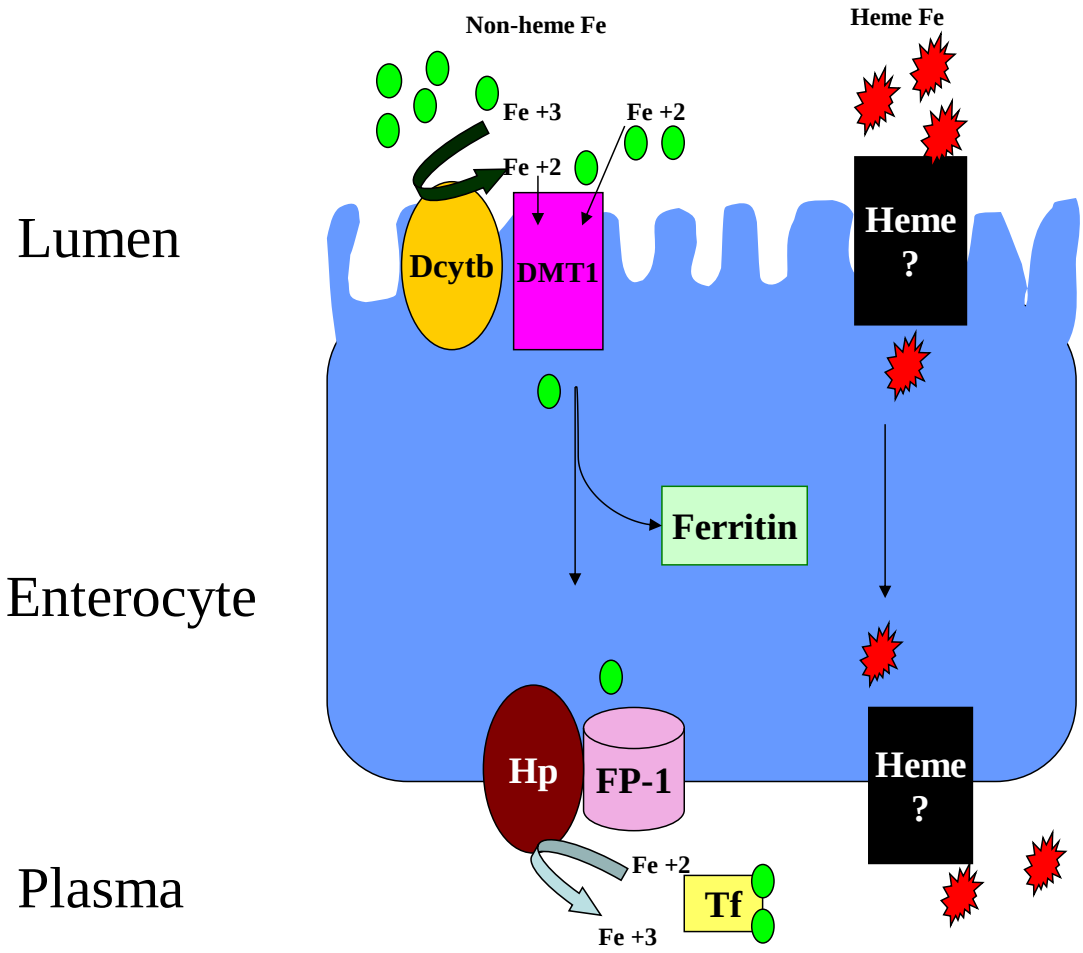

Figure 1.1.b Mechanisms of Iron Absorption II

Heme iron does not enter the inorganic iron pool in the enterocyte and is exported across the basolateral membrane intact.

Iron status of the individual and iron bioavailability have been reported to alter non-heme iron absorption up to 10 to 15 fold (29). The body has evolved a complex system of regulation of the expression of the proteins involved in cellular iron transport. In response to body iron stores and inflammation, specific iron regulatory proteins (IRPs) are up or down regulated to alter cellular iron transport (30). There are two known IRPs (IRP-1 and IRP-2) which are distinct in their regulation, tissue expression and modulation despite being structurally and functionally alike (31). These IRPs bind to specific iron response elements (IREs) located in the 3' or 5' untranslated regions (UTR) of mRNAs that encode for key iron transport proteins and allow for post-transcriptional, iron-dependent regulation. Depending on whether the IRE is located in the 3' (as found for transferrin receptor, TfR) or 5' (as found for ferritin) end of the UTR, binding of iron regulatory proteins (IRP) may stabilize or inhibit the translation of key iron proteins in response to cellular iron status. In conditions when the cell is iron-loaded, IRP-1 assembles an iron-sulfur cluster (4Fe-4S) preventing IRE binding and IRP-2 undergoes proteasomal degradation (31). This up or down regulation of target genes using the same IRP allows for coordinated shifts from production of either iron storage proteins or iron transport or export proteins in response to body needs. Using murine pro-B lymphocyte cell lines, researchers have demonstrated that IRP-2 was capable of functioning as the only IRE-dependent mediator of iron homeostasis and that regulation of ferritin and TfR expression was not dependent on IRP-1 (32). In addition, IRP-1 knockout mice did not lead to any apparent abnormal phenotypes (33), while IRP-2 knockout mice exhibit aberrant iron homeostasis and iron accumulation in the intestine and certain areas of the brain (34). However, further research on the relative roles of IRP-1 and IRP-2 is needed.

Hepcidin is a small cysteine rich peptide hormone that has recently emerged as a key regulator of iron homeostasis (35). Hepcidin, initially referred to as LEAP-1, was discovered as an urinary antimicrobial peptide synthesized in the liver (20;36) that could be induced by both high iron status and inflammation (21). Further research has also demonstrated that hepcidin expression may also be diminished in response to increased erythroid drive, hypoxia, and iron deficiency (37). Hepcidin is believed to impact iron metabolism through four primary pathways: regulation of iron absorption in the gut; iron recycling from macrophages; control of hepatic iron storage; and regulation of placental iron transport to the fetus during pregnancy (35). Hepcidin regulates iron absorption by binding to ferroportin (the basolateral iron export protein) leading to its internalization and degradation within lysosomes (38). The loss of ferroportin effectively blocks iron export from the enterocyte, leading to a reduction in intestinal absorption. Recent research also suggests hepcidin may inhibit apical iron uptake in intestinal cells, and its role in regulating iron metabolism is cell dependent (39). Due to technological issues of measuring serum hepcidin, only within the past year has a relationship between serum hepcidin expression and iron absorption in humans been demonstrated in women, Chapter 2, (40) and men (41). Although a number of factors have been identified that may influence non-heme iron absorption, to date no studies have examined both heme and non-heme iron absorption during pregnancy. This study will provide novel data on the key determinates of both heme and non-heme iron absorption.

II. Placental Iron Transfer During Pregnancy

The placenta is a unique temporary organ that functions as the fetus's lungs, kidneys, digestive system, liver, endocrine and immune system in order to ensure the growth and development of the fetus. The placenta is able to mediate the transmission

of nutrients to the fetus. Humans have a hemochorial placenta; indicating that the maternal blood is in direct contact with the fetal chorionic villi (42). In the mature hemochorial placenta there are only two layers separating maternal and fetal blood, the syncytiotrophoblast (STB), and fetal endothelial cells. The STB is able to selectively regulate transport of oxygen and essential nutrients to the fetus while also allowing for the excretion of fetal waste products (ex. carbon dioxide) to be cleared by maternal circulation.

During the third trimester of pregnancy the majority of fetal iron stores are acquired and many nutrients such as iron are actively transported across the STB against a concentration gradient. This placental iron transfer may set the stage for postnatal iron status and the risk of developing iron deficiency in infancy. The neonatal gut is immature and is not able to regulate iron metabolism so iron stores at birth are very important. Therefore, understanding mechanisms of fetal iron transport during the third trimester of pregnancy is important to ensuring adequate iron endowment at birth.

Within the placenta iron is absorbed across the microvillar membrane (also known as apical or maternal membrane), transferred across the cytoplasm of the STB, exported through the basal fetal membrane and then transported across the fetal endothelial lining before reaching the fetal circulation. Placental iron transport is depicted in **Figure 1.2**; however it is important to note that this diagram is a best estimate of iron transport and is not nearly as scientifically robust as the enterocyte model.

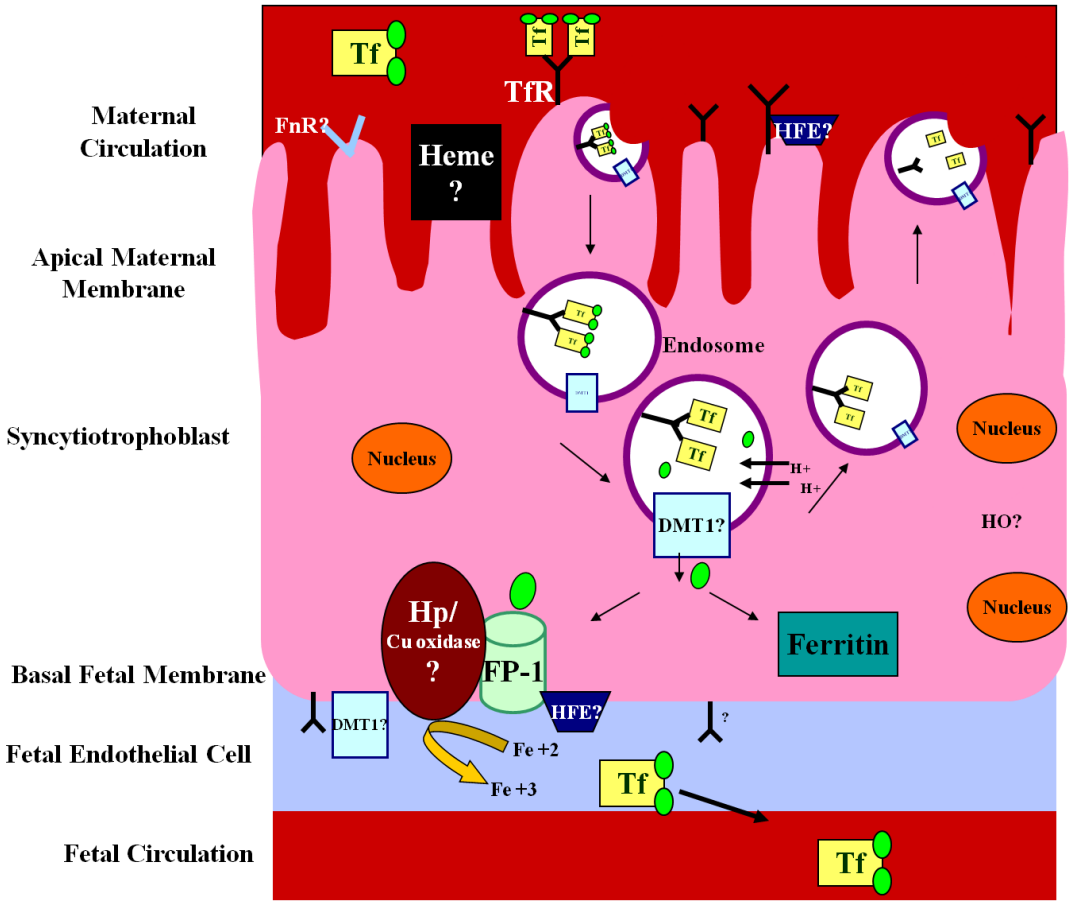

Figure 1.2 Mechanisms of Placental Iron Transport

Maternal iron is transported across the apical membrane of the syncytiotrophoblast (STB) by receptor mediated endocytosis, transferred across the cytoplasm of the STB, through the basal fetal membrane, across the fetal endothelial lining and then is released into the fetal circulation. The mechanisms by which this transport occurs remain elusive and require further investigation.

Transferrin receptor (TfR) is expressed on the apical (maternal) side of the STB membrane where it is responsible for binding maternal transferrin (43). Once bound, this transferrin-TfR complex is internalized into clathrin-coated endosomes by receptor mediated endocytosis. This process is believed to occur as previously reported for other tissues, where the pH of the endosome is decreased with a proton pump to release the iron from transferrin and the apo-Tf and TfR are recycled to the cell membrane to repeat the cycle (44). Iron is likely released from acidified endosomes into the cytoplasm via divalent metal transporter 1 (DMT1) (45).

Recent research has questioned the essentiality of DMT1 in the placenta, as murine data found that although DMT1 was vital for intestinal non-heme iron absorption after birth, DMT1 was not required for placental iron transfer (46). An alternative or complementary route for iron uptake on the apical membrane may occur via the ferritin receptor (FnR). The FnR has been identified in the placenta by one group although further research is needed to validate this finding and its importance for placental iron transfer (47). Knowledge in this field is continuing to grow and there are possible other novel undiscovered key iron transporters as well as additional repetitive iron transporters due to the importance of tightly regulating fetal iron transfer. Current research also has yet to elucidate the mechanism by which iron is transferred across the syncytiotrophblast cytoplasm from the apical to the basolateral side of the placenta. Some researchers have speculated that an unidentified chaperone protein may be involved in this transport (48). Excess iron may also be stored in the placenta as ferritin (Ft) and not transported to the fetus to prevent excess stores from being accumulated. Iron is exported out the basal (fetal) STB membrane through ferroportin (49;50). Hephaestin or a similar copper oxidase protein is required to oxidize iron (Fe^{+2}) to be released from the cells as Fe^{+3} for incorporation into fetal transferrin (48). DMT1 and TfR expression have also been found on the placental

basal membrane; however their role in transporting iron at this location has not been established (45;49). Georgieff et al., have speculated that although the net flux of iron is from mother to fetus, that the placenta may also receive iron signals from the fetus in response to fetal iron status (45). This may be in parallel to the enterocyte where the net flux of iron is from the lumen, although a small amount of iron is also taken up from the serum to influence protein expression of iron transport proteins.

Most information available on placental iron trafficking has focused only on mechanisms of non-heme iron uptake. The expression of proteins implicated in heme metabolism in the enterocyte have been identified in the placenta: HCP1 (Proton-coupled folate transporter/Heme carrier protein), BCRP (Breast cancer resistance protein), FLVCR (feline leukemia virus receptor C), hemopexin receptor and heme oxygenase (23;51-55). However, thus far none have been linked to iron homeostasis in the placenta. As previously noted, HCP1 is now believed to function primarily in folate transport (23) and may have a similar role in the placenta. BCRP although located on the apical membrane has been identified as an iron efflux protein, and Jonker et al., have proposed that its role may be in efflux of drugs that have entered the placenta back into the maternal circulation as a protective mechanism for the developing fetus (56). Heme oxygenase, while involved in heme metabolism in other tissues has a number of other functions in the placenta involving placentation, hemodynamic control and antioxidant protection (51). Researchers have yet to elucidate a pathway of placental heme iron transport. If following maternal iron absorption heme iron joins the common iron pool in the enterocyte and is thereafter processed as non-heme iron, there may not be a separate mechanism for fetal heme iron transfer in the placenta. In addition, if mechanisms of heme iron transport are identified in the placenta it does not necessarily mean that the source of heme iron is from the gut, as heme may also be derived from hemolysis or during hemoglobin

destruction. While the mechanisms of non-heme absorption have been studied in great detail, iron transport in the placenta remains largely speculative. It has been difficult to determine the essentiality of the above placental transport proteins. In a review article by Hentze et al., the authors outlined important animal models that have aided in understanding iron homeostasis (30). In this review it was interesting to note that transferrin receptor-1 and H-ferritin knockout mice are both embryonic lethal, which may imply they are essential in regulating placenta iron transport. It has also been difficult to correlate the upregulation of placental iron transport proteins with neonatal iron transfer in humans. However, the use of two oral stable isotopes in this study allows for the ability to trace heme and non-heme iron transfer *in vivo*.

II a. Regulation of Placental Fe Transfer

The mechanisms and determinates of placental iron transfer (also known as neonatal or fetal iron transfer) are largely unknown. Regulation is likely dependent on a number of factors including: maternal/neonatal iron status, placental protein upregulation, IRP activity and hepcidin expression. However, many gaps remain in our current knowledge of this intricate system.

The role of maternal iron status in determining infant iron status has been a controversial issue. Previously, the fetus was believed to be a "perfect parasite" and capable of extracting essential nutrients from the mother, regardless of maternal iron status. This theory has been supported by research findings where there has been no significant relationship between mild or moderate maternal anemia and infant iron status (57-60). Research into this relationship often relied only on measures of hemoglobin or hematocrit. Multiple iron status indicators are now available and more robust measures may identify deficits in storage iron pools. The other constraint is that most studies are only capable of sampling circulating iron stores and subtle deficits in

the brain, myelination or other storage sites of iron may not be measurable. However, now there is a growing consensus among researchers that maternal anemia does in fact impact the iron endowment at birth (61-64) and into the first 6 months (65;66). Maternal iron status has also been shown to influence maternal non-heme iron absorption and transfer to the fetus (67). How the placenta mediates this iron transfer under various conditions of maternal iron status is unclear. The placenta may extract iron from maternal circulation in proportion to maternal iron status, as a direct linear correlation with placenta iron concentration and maternal iron status has been illustrated (68). However, the research is not conclusive as other researchers have found no correlation between maternal iron status (Hb, TfR, Ft) and placental iron concentration or expression of transferrin receptor and ferritin (69).

Increased expression of placental iron proteins may serve as a compensatory mechanism to increase fetal iron transfer under conditions of iron insufficiency. Under iron replete conditions the placenta may also accumulate iron as ferritin which serves as an iron storage pool to protect the fetus against iron overload (70). However, there are limits in the placenta's ability to upregulate transport mechanisms as needed and several human conditions are known to be associated with alterations in iron stores. In diabetic women, despite a demonstrated increase in placental transferrin receptor mRNA expression infants were still born with abnormally low iron stores (as defined as cord serum ferritin concentrations less than 60 ng/ml) (71). Likewise, increased placental transferrin expression has been demonstrated in abnormal pregnancies (drug abuse, pregnancy-induced hypertension, and gestational diabetes) compared to normal pregnancies perhaps in response to altered fetal demands, although iron status measures were not reported (72). In animal models the expression of both TfR and the IRE-regulated form of DMT1 have been shown to increase in response to conditions of maternal iron deficiency (50).

Ferroportin is developmentally regulated in the placenta and mRNA levels are the highest in the third trimester of pregnancy when fetal iron transport is at its peak (73). Research using BeWo cells, a placental carcinoma cell line, has found that ferroportin expression was not changed in response to iron deficiency, however, copper oxidase activity was increased and allowed for increased iron transfer (50). Currently it is unclear whether the copper oxidase identified in the placenta is in fact hephaestin, or a similar ferroxidase. Whether the expression of ferroportin and hephaesin/Cu oxidase are altered in the human placenta in response to both maternal and fetal iron status has not been well established. The role of HFE (Human hemochromatosis protein) in placental iron transfer is also unclear. In the enterocyte, HFE is associated with TfR in the crypt cell and is proposed to be involved in the regulation and uptake of transferrin-bound iron in response to body iron stores and aid in the programming of key iron transport proteins (74). Although, HFE's primary role may not be in programming but rather in modulating the expression of hepcidin (75). Previous research has found HFE to be expressed in the apical plasma membrane of the syncytiotrophoblast (STB) cells and associated with TfR (76). This suggests that HFE could function in regulating the transfer of maternal iron to the fetus in the placenta. However, more recent data has found HFE localized on the basal STB (49). On the basal STB HFE was co-localized with ferroportin. In contrast to previous research, there was only a minimal association between TfR and HFE. In addition, the amount of HFE expressed varied with samples, which could suggest differential protein expression due to maternal/fetal iron status; however this was not directly addressed in the research and warrants further review. To date, no information exists on the ability to increase heme iron transport to the fetus. In addition, the degree to which iron transfer can be upregulated is still unclear and the key determinates of placental protein expression have yet to be fully elucidated *in vivo*.

Similar to the enterocyte, placental iron transport may also be influenced by the IRE/IRP regulatory system. Neonatal iron status (as measured by cord serum ferritin) has been found to be inversely correlated to both placental IRP-1 and IRP-2 and placental ferritin was likewise correlated with IRP-1 (77). However, in this study protein expression of placental TfR and FP-1 were not related to IRP-1, IRP-2 activity or neonatal/placental iron status. The lack of a relationship with placental iron transporters may be the result of a narrow range of iron status. In contrast, other studies have shown that placental IRP-1 activity is directly related to TfR mRNA concentration in human placenta (71). Additional research is needed to clarify the role of IRP activity in fetal iron transfer.

The role of hepcidin in regulating placental iron efflux is largely unknown. As previous described, hepcidin is a negative regulator of iron homeostasis. Hepcidin functions by degrading ferroportin which decreases serosal iron transfer in the enterocyte, inhibits the release of iron in macrophages, and blocks placental transport (78). In studies with transgenic mice over expressing hepcidin, mice died shortly after birth due to severe iron deficiency (79). In addition, in pregnant rats hepatic hepcidin mRNA was found to progressively decrease with gestation (80). This corresponded with increased expression of intestinal iron transporters (DMT1, DCYTB & FP-1) which may help explain the greater fetal iron flux during the third trimester of pregnancy. However, hepcidin expression has not been studied in relationship to iron absorption and fetal iron transfer during human pregnancy and this may provide key information on regulation of iron homeostasis.

III. Assessment of Iron Status

There are three primary stages of iron deficiency; iron depletion, iron deficient erythropoiesis and iron deficiency anemia which correspond to alterations in storage

iron, transport iron and ultimately functional iron. The World Health Organization recommends the use of multiple indicators to assess iron deficiency and advises that the best combination of markers may be hemoglobin, serum transferrin receptor and serum ferritin or bone-marrow iron in order to reflect the functional impairment, tissue avidity for iron and iron storage (81). Serum ferritin was selected due to the ease of measurement and cost. Also, bone-marrow biopsy is not an accepted method for screening in healthy populations. A basic description of iron status indicators selected for this study and their advantages and disadvantages are outlined in **Table 1.1**.

Total body iron (TBI) was calculated based on a formula developed using serial quantitative phlebotomy data in healthy men and non-pregnant women: (total body iron (mg/kg) = -[log (serum transferrin receptor/serum ferritin) – 2.8229]/0.1207) (82;83). With this formula, iron stores are expressed with positive values and negative values correspond with functional iron deficiency. From the phlebotomy experiments, negative values represent the additional amount of iron removed after stores were depleted, or in other words, the quantity of iron required before iron stores could be restored. Although validation of this equation is not possible in pregnant women and neonates, this measure has been utilized to assess iron status in pregnant populations (84;85).

Normal biomarkers of iron deficiency are difficult to interpret in infants as iron absorption and metabolism are not fully developed (86). The assessment of newborn iron stores is further complicated due to limited normative data on iron status indicators among healthy neonates and lack of well defined cut off values for iron deficiency in infancy. In addition, for both adults and infants serum hepcidin measurements are not well defined or standardized.

Table 1.1 Assessment of Iron Status[1]

Iron Status Indicator	Brief Definition	Advantage	Disadvantage
Hemoglobin (Hb)	Measure of anemia, reflect amount of functional iron in body.	Inexpensive, universally available, simple to measure	Low sensitivity/ specificity, late indicator
Hematocrit (Ht)	Packed cell volume, proportional volume of RBCs in whole blood. Reflect amount of functional iron in body.	Same as Hb	Same as Hb
Serum Transferrin Receptor (TfR)	Indicator of tissue iron availability. Reflects balance between cellular iron requirements and iron supply.	Quantitative, unaffected by inflammation	Lacks standardization, effected by the rate of erythropoiesis
Serum ferritin (Ft)	Storage iron. Reflect total body iron stores.	Quantitative, well standardized	Acute phase protein, affected by inflammation and liver disease
Total Body Iron (TBI)	TBI (mg/kg) = -[log (serum transferrin receptor/serum ferritin) – 2.8229]/0.1207	Measure of full range of iron status, validated by phlebotomy studies in adult volunteers	Same as components, equations not validated in pregnant women
Hepcidin	Regulator of iron homeostasis.	Production diminished when iron reserves depleted	Assay methods and interpretations of results are under development

[1]Adapted from (81;87;88)

There is no gold standard for assessing serum hepcidin and it has been difficult to access due to its small size (25AA) and unique folding structure. At present there are several methods which include, the new serum hepcidin competitive enzyme-linked immunosorbent assay (89), surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) (90;91), liquid chromatography tandem mass spectrometry (92) and the commercially available ELISA for serum prohepcidin (93-95). It remains difficult to compare values among studies using different methods due to the lack of standardization and further research is needed to validate each of these methods. Furthermore, data analysis and interpretation are complicated by the fact that iron deficiency decreases serum hepcidin often to levels below the limit of detection.

Despite the limitations in assessment, the use of the above iron status indicators in combination provides meaningful values to compare the relative differences among subject participants in our study.

IV. Stable Isotope Methods

Stable isotopes provide a valuable tool for examining iron bioavailability and metabolism (96). Stable isotopes have been used in clinical research for several decades without any significant biological side effects and are considered appropriate for use in pregnant women and children (97;98). Iron stable isotopes are non-radioactive forms of iron that are naturally found in fixed amounts in our body and in the environment. Iron in nature is comprised of 4 stable isotopes ^{54}Fe, ^{56}Fe, ^{57}Fe and ^{58}Fe. Three of these forms of iron (^{58}Fe and ^{57}Fe and ^{54}Fe) are found at very low levels in our environment (0.287%, 2.14% and 5.8% respectively) and enriched sources of these stable isotopes can be utilized to trace maternal iron absorption and placental iron transfer. Since the majority (80-90%) of newly absorbed iron is taken up by red blood cells (RBC) for

hemoglobin synthesis, iron absorption can be assessed by determining the fraction of the isotope that is incorporated into red blood cells. The use of magnetic sector thermal ionization mass spectrometry (TIMS) enables the precise measurements of isotopic ratios. In brief, iron is isolated from the blood samples and dried onto rhenium filaments. The filaments are heated slowly leading to the evaporation and ionization of the iron. The ionized atoms are then mass-separated by an electromagnet (the lighter isotopes are deflected more and the heavier isotopes are deflected less). The separate beams for each isotope are collected in Faraday cups that convert the ion beam into a measurable voltage whose output is tracked and quantified using computer programs. A ratio is made between each administered iron isotope and another iron isotope that has not been given (^{56}Fe). Calculations are adjusted for isotopic fractionation by correcting measured voltages to the expected ratio of two non-administered iron isotopes (^{54}Fe/^{56}Fe). Fractionation refers to the tendency of lighter isotopes to evaporate from filaments at faster rates than heavier isotopes and is automatically corrected for by the specialized computer program that provides the normalized isotopic ratios of the samples. The degree to which the normally occurring amounts of these isotopes in the body are altered following dosing can then be utilized to determine iron absorption. Relative standard deviations using this method average 0.015% and 0.16% for the $^{57/56}$Fe and $^{58/56}$Fe ratios, respectively. Detailed calculations are discussed in further detail in the methods section.

Common methods for administering stable iron isotopes include dosing with one or two oral isotopes or one oral and one intravenous (IV) isotope. The use of an IV tracer allows for the measurement of red blood cell (RBC) incorporation directly. When two oral isotopes are used estimates of RBC iron incorporation must be used. Common estimates are 80% for adults and 80- 90% for children and these estimates are very stable in healthy populations (99). During pregnancy this estimation leads to

more uncertainties as RBC iron incorporation is significantly more variable during pregnancy due to increases in the size of the intravenous distribution pool and other physiologic changes of pregnancy. The magnitude of RBC iron incorporation during pregnancy has been found to be affected by iron supplementation and maternal iron status (serum ferritin) (13). A limitation of our current study is that we will not use an IV tracer. However, since this is a constant factor in our estimates it will not impact relative differences between measurement of heme and non-heme iron absorption or transfer.

Although the use of one oral and one IV tracer has advantages a limitation with this approach is that iron absorption may only be determined from one iron source. The use of two oral tracers enables comparisons in the bioavailability of two iron sources. Each subject serves as their own control and differences in RBC iron incorporation should cancel out in the same subject. It is not possible to give two oral tracers and one IV tracer as two non-enriched isotopes are necessary to correct for fractionation.

Oral stable isotopes can be used as either intrinsic or extrinsic labels to measure nutrient absorption (100). An intrinsic label involves the biological incorporation of the isotope into the food administered. Extrinsic labeling involves mixing the stable isotope with the food before consumption, based on the assumption that the tracer and the native inorganic element will be absorbed and metabolized in the same fashion. Research by Cook et al., has validated the use of extrinsic tags for several foods and found highly consistent non-heme iron absorption values from foods such as maize, black bean, and wheat studied with either extrinsic or intrinsic labeling (101).

Heme iron on the other hand, is absorbed through a separate pathway than non-heme iron and is best examined with an intrinsically labeled tracer. Until recently,

stable isotope techniques for determining heme iron absorption had not been developed and only radioisotopes had been utilized (102). To determine heme iron absorption, several studies have injected radioactive iron (^{59}Fe or ^{55}Fe) into anemic rabbits to create biosynthetically labeled rabbit hemoglobin (103-106), while others have used radio-labeled animal tissue or a combination of radio-labeled hemoglobin and/or animal tissue (107). Martínez-Torrez et al. intravenously injected ^{55}Fe into 3-month old calves and found that most of the total radioactivity (95%) in veal muscle was present as heme compounds (hemoglobin and myoglobin) (107). Thus either the red blood cells or muscle tissue of intrinsically labeled animals may be fed to humans to examine heme iron absorption. While these radioisotope studies have provided key information on heme metabolism, due to the risks involved with radioisotopes during pregnancy they cannot be applied to investigate human iron transfer to the fetus (108). Etcheverry et al. developed a method to successfully infuse stable iron isotopes (^{58}Fe) into an anemic calf to produce enriched RBCs which could then be used to trace heme iron absorption safely in children (102). Working in collaboration with this research group we will use a modified version of their method. This research project is the first study to utilize intrinsically labeled meat and red blood cells to examine heme iron absorption and fetal iron transfer during pregnancy.

Rationale and Summary

In summary, while non-heme iron metabolism in the enterocyte has been well established, the pathway of placental non-heme and heme iron absorption remains largely unknown. In addition, the mechanisms and determinates of placental iron transfer remain uncertain. The placental proteins and mechanisms involved in the regulation of these proteins have not been well characterized. It has been difficult to correlate these processes with transfer of iron to the neonate *in vivo* in humans due to

the lack of suitable methods. Dual stable iron isotopes allow for simultaneous tracking of heme and non-heme utilization so the differential determinants of each can be characterized. By following the enrichment of stable iron isotopes, *in vivo* heme and non-heme maternal iron absorption and partitioning of heme and non-heme iron to the neonate can be examined in relation to maternal and neonatal iron status and serum hepcidin. These data provide novel information on the dynamics of iron metabolism during pregnancy and in non-pregnant women of childbearing age.

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CHAPTER 2

SERUM HEPCIDIN IS SIGNIFICANTLY ASSOCIATED WITH IRON ABSORPTION FROM FOOD AND SUPPLEMENTAL SOURCES IN HEALTHY YOUNG WOMEN*

*** Young MF, Glahn RP, Ariza-Nieto M, Inglis J, Westerman M, O'Brien KO. Serum hepcidin is significantly associated with iron absorption from food and supplemental sources in healthy young women. Am J Clin Nutr 2009;89:533-8.**

Abstract

Background: Hepcidin is a key regulator of iron homeostasis but to date no studies have examined the impact of hepcidin on iron absorption in humans.

Objective: To assess relationships between both serum hepcidin and serum prohepcidin with non-heme iron absorption assessed in the presence and absence of food using dual stable iron isotope techniques.

Design: The study group included 18 healthy, non-pregnant women. Women received in random order: a supplemental iron source (8.5 mg of ferrous sulfate containing 0.9 mg of ^{58}Fe as ferrous sulfate) and 6.8 mg of ^{57}Fe ferrous sulfate tracer administered with a non-heme food source (orange fleshed sweet potato; OFSP: 1.4 mg native iron). Iron absorption was determined by analyzing blood samples taken 14 days after dosing using magnetic sector thermal ionization mass spectrometry. Serum hepcidin was assessed by a new competitive serum enzyme-linked immunosorbent assay specific for the refolded, mature 25 amino acid form and serum prohepcidin was assessed by an ELISA specific for amino acids 28-47 of the hepcidin prohormone.

Results: In these women iron absorption averaged 14.71 ± 10.7% from the supplemental iron compared to 3.63 ± 6.5% from the OFSP. Absorption of non-heme iron assessed in the presence ($p = 0.038$, $r^2 = 0.241$) d absence of food ($p = 0.0296$, $r^2 = 0.263$) was significantly associated with serum hepcidin but was not significantly related to serum prohepcidin.

Conclusions: Serum hepcidin, but not prohepcidin, was inversely associated with iron absorption from supplemental and food based non-heme iron sources in iron replete healthy adult females.

Introduction

Hepcidin has recently emerged as a key regulator of iron homeostasis (1). This protein is a small cysteine rich peptide hormone produced in the liver and is measurable in human urine (2) and plasma (3). Hepcidin is believed to impact iron metabolism via regulation of iron absorption in the gut; iron recycling from macrophages; control of hepatic iron storage and this hormone also appears to regulate iron transfer through the placental syncytiotrophoblast during pregnancy (1). In the enterocyte, hepcidin binds to the basolateral iron export protein, ferroportin, initiating its internalization and degradation, effectively blocking iron flux from the cell and reducing iron absorption (4). Over expression of hepcidin has been associated with anemia of inflammation (5;6) and severe iron deficiency anemia in transgenic mice (7). On the other hand, considerably reduced hepcidin expression occurs in patients with hereditary hemochromatosis (8;9).

Although hepcidin expression has been found to be inversely associated with iron absorption and expression of iron transport proteins in rats (10), to date a significant relationship between hepcidin expression and iron absorption in humans has not been established. This has been in part due to the small size of this protein (25 amino acids, AA) and the inherent difficulties associated with its measurement. This has led some researchers to measure prohepcidin, a linear 60-AA precursor to the mature, refolded, 25-AA peptide containing four disulfide bonds. At present the validity of using prohepcidin as an index of hepcidin expression has been questioned due to the inability to find an association between prohepcidin and iron absorption (11;12).

The objective of this study was to assess relationships between serum prohepcidin and hepcidin concentrations with iron absorption using dual stable iron isotope techniques. A second objective was to compare the strength of these

relationships from a food based matrix versus that observed from a supplemental source of iron (ferrous sulfate). We hypothesized that iron absorption would be more significantly associated with serum hepcidin versus prohepcidin and that the strength of these relationships would be higher from supplemental versus food based non-heme iron sources.

Subjects and Methods

Subjects

Eighteen young women ages 18-32 years were recruited into the study beginning in the spring of 2007. Subjects were eligible for the study if they were not taking any vitamin or mineral supplements and did not plan to ingest any over the 2-week study interval. Subjects were also questioned on their previous supplement use and none of the subjects had taken supplements for at least one month prior to the study. None of the subjects had a history of intestinal or malabsorption problems, blood disorders, ulcers, or joint disease and none were taking any prescription medications known to impact iron homeostasis. Informed written consent was obtained from each subject and the study was approved by the Institutional Review Board of Cornell University.

On the morning of the study, fasted subjects were admitted to the Human Metabolic Research Unit (HMRU) at Cornell University. Upon arrival, the women's height and weight were taken (in street clothes and without shoes) with the use of a stadiometer and calibrated scale. On the first day of the study women were randomized to receive either ferrous sulfate alone or combined with a non-heme food based iron source (orange fleshed sweet potato - OFSP). The OFSP used for this study were shipped from Peru and were baked in the oven at 350° until soft. Once cooked, the skin was removed from the potatoes and they were mixed thoroughly. Individual servings

were frozen until use and briefly microwaved before serving. The non-heme food based meal consisted of approximately 240 g of OFSP [*Ipomoea batatas* (L.) Lam.] to which 6.8 mg of ^{57}Fe tracer as ferrous sulfate was added to obtain a total iron content from the potato and tracer of approximately 8.2 mg. The sweet potatoes were served alone and only pepper was used for additional flavoring if desired. The supplemental iron consisted of 7.6 mg of ferrous sulfate (0.9 mg of which was ^{58}Fe tracer as ferrous sulfate). The supplement was given with 1.5 ml of flavored syrup composed of a liquid sugar substitute (Superose, Medina, NY) flavored with two to three drops of orange extract (McCormick Hunt Valley, MD) and administered orally by a syringe. The only beverage allowed during consumption of meals was water.

Total iron content of each iron load was measured with atomic absorption spectrophotometry (AAS, Perkin Elmer Analyst 800, Norwalk, CT). Calcium content of the OFSP was measured using a coupled argon plasma emission spectrometer (ICP-ES) (ICAP Model 61E Trace Analyzer, Thermo Jarrell Ash Corporation, Waltham, MA).

After consuming each food, subjects remained in the HMRU for the following two hours before being fed a light snack (granola bar) then after an additional two hours subjects consumed a standardized lunch (vegetable soup, pretzels, and water). The following day the women came back to the HMRU at the same time and the study was repeated using the second of the randomized foods and following the same study design and snack and lunch meals. Two weeks after the second iron tracer dose was ingested the subjects returned to the HMRU and a 7.5 ml venous plasma and 7.5 ml venous serum sample were obtained.

Laboratory Analysis

Serum ferritin was measured by a commercially available enzyme immunoassay procedure (Ramco Laboratories, Inc Stafford Texas). Serum soluble

transferrin receptors (TfR) were measured with an enzyme linked immunosorbent assay (Ramco Laboratories, Inc Stafford Texas). Total body iron was calculated by the ratio of serum transferrin receptor to serum ferritin as described by Cook et al.; (total body iron (mg/kg) = -[log (serum transferrin receptor/serum ferritin) – 2.8229]/0.1207) (13). Hemoglobin was analyzed using a HemoCue (Lake Forest, CA). Serum folate, vitamin B_{12} and C-reactive protein (CRP) were measured using the Immulite®1000 immunoassay system (Tarrytown, NY). Serum prohepcidin was measured by a solid phase enzyme-linked immunosorbent assay with antibodies specific for peptides 28-47 of the hepcidin prohormone molecule (DRG International Inc., Mountainside NJ). Blinded serum samples were analyzed by Intrinsic Life Sciences to measure serum hepcidin (Intrinsic Life Sciences, La Jolla California) using a newly developed competitive serum enzyme-linked immunosorbent assay (C-ELISA) specific for the mature peptide. Detailed methods for the performance of this assay have recently been published (14). Using this method; typical intra-assay precision and inter-assay coefficients of variations achieved are 5-19% and 12% respectively. Moreover, this assay has also been shown to appropriately reflect alterations in iron homeostasis (14).

Isotope preparation and sample analysis

Iron isotopes (^{57}Fe at 88% enrichment and ^{58}Fe at 93% enrichment) were purchased as the metal from Trace Sciences International (Ontario, Canada). Both oral tracers were converted into sterile, pyrogen-free solution of ferrous sulfate by Anazao Health Corporation (Tampa, FL). The isotopic composition of the tracer solutions was validated using a Thermoquest Triton TI Magnetic Sector Thermal Ionization Mass Spectrometer (Breman, Germany). Whole blood samples (0.5 mL) were digested with 4 mL concentrated Ultrex nitric acid in a Teflon beaker. Samples were then dried down

overnight on a hotplate at 80°C and redissolved in 7 M ultrapure hydrochloric acid (HCl) (Ultrex II; J.T. Baker, Phillipsburg, NJ). Iron was extracted using a modified anion exchange chromatography method based on that previously described (15;16). Small plastic columns were filled with anion exchange resin (AG 1-X8, Bio-Rad Laboratories, Hercules, CA) in deionized (DI) water. The column was washed with the following sequence: twice with DI water, once with 0.5 M ultrapure HCl, twice with DI water, and four times with 7 M ultrapure HCl. Digested blood samples in 7 M ultrapure HCl solution were then added to the column and 7 M ultrapure HCl was added drop wise to the columns before collecting the eluted iron with the drop wise addition of 0.5 M ultrapure HCl. The iron samples were dried on the hot plate and were reconstituted in 50 μL of 3% HNO_3.

Extracted iron samples (8 μL) were loaded onto a rhenium filament (H. Cross Company Weehawken, NY) with 4 μL of silica gel (Sigma-Aldrich, Inc., St. Louis, MO) and 4 μL of phosphoric acid (0.7 N). Isotopic ratios of $^{57/56}$Fe and $^{58/56}$Fe were measured and ratios were normalized to the $^{54/56}$Fe ratio. The fractional abundance values utilized were 0.02317 for ^{57}Fe and 0.00308 for ^{58}Fe. Relative standard deviations obtained averaged 0.015% and 0.16% respectively for the $^{57/56}$Fe and $^{58/56}$Fe ratios, respectively.

Calculations

Iron absorption was calculated using previously described methods (17;18). The quantity of ^{57}Fe and ^{58}Fe incorporated into erythrocytes (Fe_{inc}) was determined by enrichment and total circulating iron (Fe_{circ}) which was estimated by using a mean blood volume for women (70 mL/kg), the concentration of iron in the hemoglobin (3.47 g/kg), the subjects' hemoglobin value (g/L) and weight (kg). The final calculation for iron absorption was determined based on the assumption that 80% of

the absorbed isotope was incorporated into erythrocytes. An additional correction factor was also used in the analysis to take into account the small amount of ^{58}Fe in the ^{57}Fe tracer (18).

Data analysis

All statistical analyses were completed using the STATVIEW 5.0.1 software program (Abacus Concepts, Berkeley, CA). Paired t-tests were used to determine the significance of relationships between the each iron source and iron absorption. Simple linear regression analysis was used to determine relationships between iron status (serum ferritin, transferrin receptor, total body iron and hemoglobin), iron absorption, serum prohepcidin and serum hepcidin. Data distributions were viewed by examining the normal quantile plots and histograms of the data and normality was assessed using the Goodness-of-fit test (Shapiro Wilk W test) using JMP 7.0 (Cary, NC). Normally distributed data are presented as the mean ± SD and data not normally distributed (^{57}Fe absorption, age, serum ferritin, CRP, and serum hepcidin) are presented as the geometric mean ± SD. Variables that were not normally distributed (serum hepcidin, ^{57}Fe absorption, CRP and serum ferritin) were transformed by using a natural logarithm prior to analysis for statistical purposes. Results shown are the non-transformed data for interpretation purposes. Results were significant if $p < 0.05$.

Results

Subject characteristics

General characteristics of the study subjects are shown in **Table 2.1**. All subjects had folate and B_{12} status within normal ranges (defined as > 5 ng/ml and > 200 pg/ml, respectively). Two subjects had elevated CRP values (15 and 12.1 mg/L).

Table 2.1
General characteristics and iron status indicators of the 18 study participants

Variable	Mean ± SD	Range
Age (y) [1]	22.3 ± 3.1	18.0 - 32.0
Weight (kg)	60.2 ± 8.2	47.0 - 78.2
BMI (kg/m^2)	22.8 ± 2.8	18.6 - 30.3
Hemoglobin (g/dL)	12.6 ± 1.3	11.0 - 15.6
Folate (ng/ml)	18.4 ± 5.5	11.5 - 31.9
Vitamin B-12 (pg/ml)	594.6 ± 255.5	241.0 - 1088.0
C-reactive protein (mg/L) [1]	0.95 ± 4.4	<0.2 - 15
Serum ferritin (μg/L)[1]	27.8 ± 34.1	5.7 - 119.7
Serum TfR (mg/L)	4.7 ± 1.3	2.8 - 7.1
Total body iron (mg/kg)	5.05 ± 3.4	-1.5 - 11.7
Serum prohepcidin (μg/L)	47.0 ± 12.9	30.7 - 77.8
Serum hepcidin (μg/L) [1]	39.95 ± 69.6	1.5 - 248.5

[1] Geometric mean

However these subjects did not have elevated serum ferritin values, and all serum ferritin values were within a normal range reported for healthy young women (19). Excluding individuals with elevated CRP from subsequent analyses did not significantly alter study results so these subjects were included in all remaining analyses. There was a limited range of iron status among these healthy participants with the majority being non-anemic, as evidenced by the finding that none had a hemoglobin value less than 11 g/dL (the mean hemoglobin value in the group was 12.6 ± 1.3 g/dL; a value above the cutoff of 12 g/dL used to define anemia in non-pregnant women); and all subjects had serum transferrin receptor concentrations less than 8.5 mg/L, indicating the absence of tissue iron deficiency (20). Only one of the 18 subjects had a total body iron level below zero (indicative of depleted iron reserves) and this subject also had a serum ferritin concentration <12 μg/L. There was a mean difference of 23 μg/L between the serum ferritin concentrations of subjects with Hb concentrations < 12 g/dL compared to those ≥ 12 g/dL (23.9 ± 10.2, n = 7 vs. 46.9 ± 41.0, n = 11) although this difference was not statistically significant ($p = 0.17$). Relationships among iron status indicators were examined. From these analyses the only significant relationship observed was between serum transferrin receptor and the natural log of serum ferritin ($r^2 = 0.458$, $p = 0.002$).

Serum prohepcidin ranged from 30.7 to 77.8 μg/L (47.0 ± 12.9 μg/L) and was not significantly correlated with either the natural log of serum ferritin, serum soluble transferrin receptor, total body iron, hemoglobin or the natural log of CRP. Moreover, no significant relationships were observed between serum prohepcidin and the natural log of serum hepcidin ($p = 0.93$). Serum hepcidin ranged from 1.5 to 248.5 μg/L (39.95 ± 69.6 μg/L). The natural log of serum hepcidin was significantly associated with the natural log of CRP concentration ($p = 0.009$). This relationship held even when the two subjects with elevated CRP were excluded from the analysis ($p = 0.009$).

The natural log of serum hepcidin was not significantly correlated with BMI or measures of iron status (natural log of serum ferritin, serum soluble transferrin receptor, total body iron or hemoglobin).

Iron absorption

Iron absorption from ferrous sulfate administered in the fasted state was significantly greater than that measured in combination with a non-heme food based iron source ($p = 0.0006$, mean difference of 8.97%). No significant differences in iron absorption were found as a consequence of the order of feeding OFSP versus supplemental iron alone. Women absorbed an average of 14.71 ± 10.7% (0.58 - 43.9%) from the ^{58}Fe -ferrous sulfate compared to 3.63 ± 6.5% (1.4 - 22.1%) from the OFSP (non-heme food source) labeled with ^{57}Fe. The intrinsic iron content of the OFSP variety used in this study was 6.153 μg/g ± 0.6 (n = 5) and the calcium content of the OFSP was 304 ± 21 μg/g (n = 3). Due to environmental, genotypic and other factors, iron concentration in OFSP can vary widely but the iron content of the OFSP we used is comparable to that reported from a similar source (9 μg/g) using earlier data from the Food and Agriculture Organization (21).

Despite the limited number of iron deficient subjects there were inverse non-significant trends between the natural log of serum ferritin and absorption of ferrous sulfate ($p = 0.08$) and between natural log of serum ferritin and the natural log of iron absorption from the OFSP ($p = 0.12$). The exclusion of the two subjects with elevated CRP from these analyses did not change the significance of the relationship ($p = 0.09$, $p = 0.13$, respectively). The natural log of serum hepcidin was significantly correlated with both the absorption of supplemental ferrous sulfate given alone ($p = 0.0296$) or

from the natural log of iron absorption when tested in the presence of the OFSP ($p = 0.038$), **Figure 2.1**. This relationship also remained significant when the two subjects with elevated CRP were excluded from the analysis ($p = 0.028$, $p = 0.011$, respectively).

In contrast, serum prohepcidin was not significantly correlated with iron absorption from the ferrous sulfate administered alone ($p = 0.776$) or in combination with OFSP ($p = 0.429$) tested as the natural log of iron absorption from OFSP. Other iron status indicators including total body iron, serum transferrin receptor and hemoglobin were not significantly related to iron absorption from ferrous sulfate administered alone or in combination with OFSP.

Discussion

This study adds to the growing body of literature affirming the role of hepcidin as a key iron regulatory hormone and to our knowledge is the first study to assess the relationship between this 25-AA hormone and measures of iron absorption in humans. Using this approach, iron absorption from ferrous sulfate administered to fasted subjects alone or in combination with a non-heme food source were both inversely associated with the mature 25-AA form of serum hepcidin in healthy young women. In contrast, iron absorption from the food based or supplemental iron source used in this study was unrelated to serum prohepcidin, a finding that is similar to other non-significant associations published in the literature (11;12). Hepcidin is initially synthesized as a linear 84-AA preprohepcidin protein containing a putative 24-AA signal sequence, a 35-AA pro-region and a 25-AA active hepcidin peptide (1). Research by Valore et al., illustrates that the precursor protein undergoes two cleavages, first the signal sequence is lost and then the pro-region is cleaved by the hepatic prohoromone convertase furin (22).

Figure 2.1 Non-heme Iron Absorption and Serum Hepcidin.

In a group of eighteen healthy non-pregnant women iron absorption was assessed in blood samples collected 2 weeks post-dosing with ^{58}Fe (as ferrous sulfate) and ^{57}Fe (added extrinsically to a meal of orange fleshed sweet potato-OFSP). Serum hepcidin was significantly inversely related with both iron absorption from the ^{58}Fe labeled ferrous sulfate; $p = 0.0296$, $y = 31.781 – 4.628x$, $r^2 = 0.263$, $n = 18$ and with iron absorption from the ^{57}Fe meal of OFSP; $p = 0.038$, $y = 2.697 – 0.381x$, $r^2 = 0.241$, $n = 18$. A second axis has been added to the figures for interpretation purposes of transformed data.

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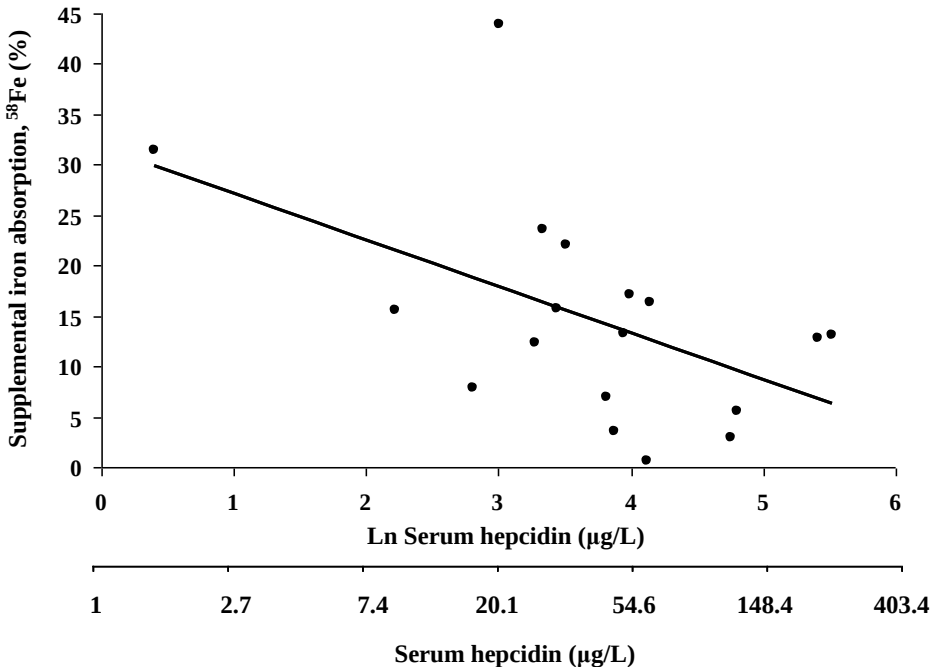
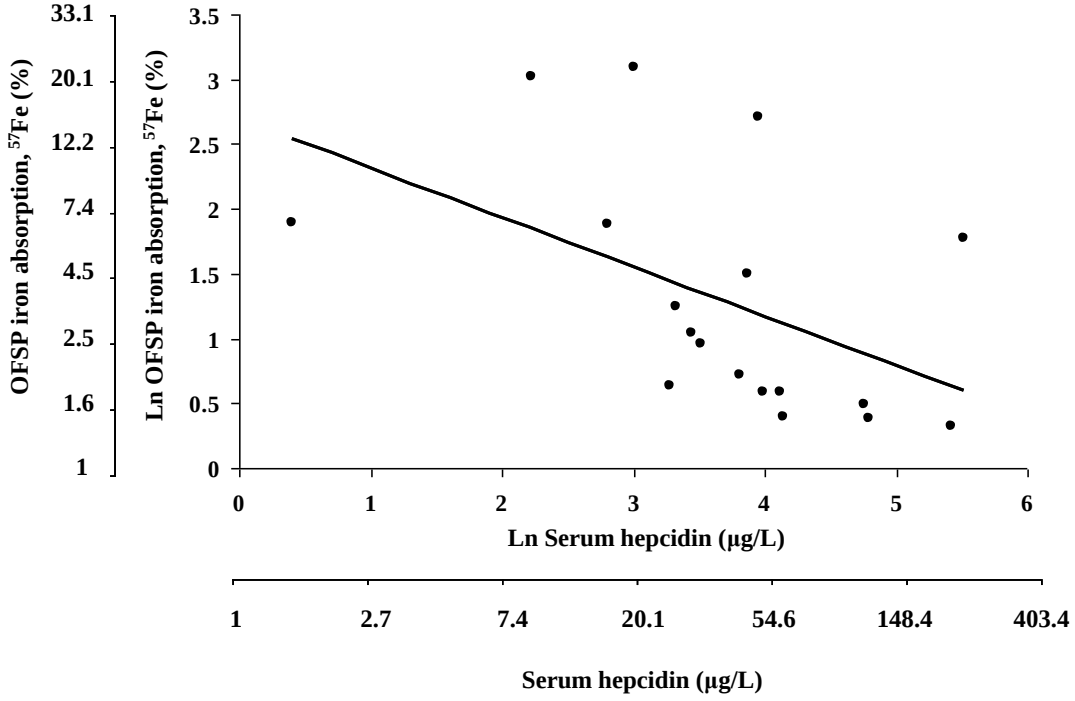

Supplemental iron absorption, 58Fe (%)
45
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
Ln Serum hepcidin (µg/L)
1
2.7
7.4
20.1
54.6
148.4
403.4
Serum hepcidin (µg/L)


OFSP iron absorption, 57Fe (%)
33.1
20.1
12.2
7.4
4.5
2.5
1.6
1
Ln OFSP iron absorption, 57Fe (%)
3.5
3
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
6
Ln Serum hepcidin (µg/L)
1
2.7
7.4
20.1
54.6
148.4
403.4
Serum hepcidin (µg/L)

In addition, there appears to be no consistent ratio or relationship between the refolded, mature form of hepcidin and prohepcidin concentrations and factors that impact hepcidin such as iron and hypoxia have no response on the cleavage of prohepcidin (22). In the present study, serum prohepcidin ranged from 30.7 - 77.8 μg/L which is a lower range than previously reported in healthy individuals by Hadely et al. (99 - 376 μg/L, n= 28 women) (11) and by Kulaksiz et al. (51.6 - 153.4 μg/L, n= 26 men and women) using the same approach (23). We found no significant relationships between serum prohepcidin, iron absorption or related indicators of iron status. This is consistent with previous research by Roe et al., that reported no significant association between prohepcidin and iron stores nor did these concentrations differ between pregnant women, patients with hereditary hemochromatosis and healthy men (12). In a similar population of healthy non-pregnant women, no significant relationships between iron absorption and serum or urinary prohepcidin were observed, leading the authors to propose that serum prohepcidin may not reflect the refolded, active form of hepcidin responsible for regulating iron absorption (11).

Serum hepcidin in these women ranged from 1.50 - 248.5 μg/L and was predominately within previously reported 5^{th} - 95^{th} percentile serum hepcidin ranges in men (29-254 ug/L) and women (17-286 ug/L) using the same serum ELISA (14). However, in other reports using liquid chromatography tandem mass spectrometry a much lower range of serum hepcidin was reported in healthy men (< 1.0 – 19.8 μg/L) and women (1.5- 45.6 μg/L) (24). Serum hepcidin was significantly associated with iron absorption in our study population. From limited data available at present; a 10 fold lower serum hepcidin concentration has been reported in anemic versus non-anemic individuals (25). Using our observed relationship between hepcidin and iron absorption, a 10 fold decrease in the mean serum hepcidin (from 66.7 to 6.67 μg/L)

would be expected to increase iron absorption by approximately 2 fold; i.e from 12.34% to 23% for ferrous sulfate alone and from 3.0% to 7.2% when administered with the OFSP source as fed in this study. However, this interpretation is limited by our small sample size and narrow iron status range. Further research is needed to understand the relationship between serum hepcidin and non-heme iron absorption in different population groups with a wider range of iron status in order to better characterize this relationship. Since this finding was reported other studies have replicated these data and reported a similar relationship between hepcidin and non-heme iron absorption (26;27).

No significant relationships between hepcidin and other measured indicators of iron status (serum ferritin, total body iron, serum transferrin receptor or hemoglobin) were observed in the current study. This finding may be due in part to the small sample size and limited number of iron deficient study participants. In other published data serum hepcidin was significantly related to serum ferritin but not to hemoglobin, serum iron, or serum transferrin receptor (28). In the current study serum hepcidin was directly associated with CRP. Two subjects had elevated CRP which may be in response to recent infection or inflammation; however all findings remained significant with or without the inclusion of these two subjects.

Iron absorption from the iron supplement (14.71%) was significantly higher when given without additional non-heme iron as OFSP (geometric mean 3.63, mean 5.75%). Our value of iron absorption from an 8 mg dose of ferrous sulfate was similar to that reported for ferrous sulfate (60 mg) by Olivares et al., in women who were studied before and after iron supplementation (9.7% and 12.5%, after adjustment to a reference dose) (29). Our value of iron absorption studied in the presence of OFSP is comparable to that reported in a group of iron replete women with similar ferritin status (6.26% and 42.6 μg/L, respectively) (30). The lower iron absorption from the

OFSP meal relative to iron supplement is likely due to the presence of the food matrix, as the components of the OFSP may affect solubility, gastric pH and absorption.

OFSP was selected as the non-heme iron source due to the importance of this staple crop in many areas of world. HarvestPlus has identified the sweet potato as one the six target crops for biofortification based on its potential to help alleviate micronutrient deficiencies in developing countries (31). In particular the OFSP is a rich source of β-carotene (32) and has been proven effective in improving vitamin A status in children (33;34). Although the iron content in OFSP is relatively low compared to animal food sources, due to its widespread daily consumption it may help contribute to daily iron needs. In this study a 240 g serving of this vegetable would provide approximately 0.1 mg of absorbable iron which corresponds to almost 7% of the estimated average daily amount of absorbed iron required (1.5 mg) by non pregnant women (35). However, it is important to note that these results were obtained from a single meal comprised only of OFSP and additional dietary factors ingested from a mixed meal may influence its bioavailability. In addition, the amount of ^{57}Fe required to trace iron absorption from this meal is considerably higher than the iron load that would be ingested by a meal containing only OFSP. Intrinsic labeling techniques or multiple additions of smaller iron tracer doses on subsequent days would be needed to more precisely assess iron bioavailability from this food source.

At present there are multiple methods that can be utilized to measure hepcidin and prohepcidin. A commercially available ELISA is available to measure prohepcidin in urine and serum samples (11;12;36). In addition to the competitive serum ELISA utilized for this study, hepcidin can also be measured by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) (25;37), liquid chromatography tandem mass spectrometry (LC-MSMS) (24) and a laboratory developed immunodot assay for urinary hepcidin (5). SELDI-TOF-MS and LC-

MSMS have limitations on equipment expense and availability and SELDI-TOF-MS also has limited analytic sensitivity and ability detect low levels of serum hepcidin (37). Interpretation of the values reported to date are confounded by the different ranges reported in these techniques and the lack of a reference material to allow for standardization of values obtained between analytical approaches and laboratories.

Iron deficiency remains the most common nutrient deficiency in the world (38). Deficiency of this mineral is increasingly recognized to have long-term and irreversible effects during key life stages including fetal development, early childhood and pregnancy (39). In spite of the importance of this nutrient many key aspects of its regulation are unanswered. These data generated using a new immunoassay, specific for the mature, bioactive form of hepcidin, present novel findings on the inverse relationship between serum hepcidin and iron absorption from non-heme iron administered as a supplement with or without the presence of a non-heme food iron source in a group of healthy non-pregnant women. Due to the role of iron in human health, continued research on the role of hepcidin and its impact on whole body iron kinetics of both heme and non-heme iron are needed in relation to age, physiological state and iron status.

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CHAPTER 3

SERUM HEPCIDIN IS NEGATIVELY ASSOCIATED WITH NON-HEME IRON ABSORPTION AND UNRELATED TO HEME IRON ABSORPTION IN PREGNANT AND NON-PREGNANT WOMEN[‡]

[‡] **Melissa F. Young, Ian Griffin, Eva Pressman, Allison W. McIntyre, Elizabeth Cooper, Thomas McNanley, Z. Leah Harris, Mark Westerman, Kimberly O. O'Brien. Serum hepcidin is negatively associated with non-heme iron absorption and unrelated to heme iron absorption in pregnant and non-pregnant women. Submitted to Journal of Nutrition.**

Abstract

Background: Heme iron absorption during pregnancy remains largely unexplored and the role of hepcidin in regulating dietary heme iron absorption is unknown.

Objective: The objective of this research was to examine the impact of hepcidin on heme and non-heme iron absorption.

Design: This study was undertaken in a group of 18 pregnant women (ages 16-32 y; week 32-35 of gestation) and 11 non-pregnant women (ages 18-27 y). Fasted subjects were randomly assigned to receive both a heme meal (intrinsically labeled ^{58}Fe pork) and labeled ferrous sulfate (^{57}Fe) fed on alternate days. Blood samples obtained two weeks post-dosing were used for assessment of red blood cell incorporation of iron isotopes, iron status and serum hepcidin.

Results: Heme iron absorption was significantly greater than non-heme iron absorption in both pregnant (47.7 ± 14.4 vs. 40.4 ± 13.2%) and non-pregnant subjects (50.1 ±14.8 vs. 15.3 ± 9.7%). During the third trimester of pregnancy absorption of non-heme iron was significantly associated with measures of iron status (transferrin receptor ($p = 0.003$), total body iron ($p = 0.02$), and hemoglobin ($p = 0.04$)). In contrast, heme iron absorption was not influenced by maternal iron stores but was inversely associated with age ($p = 0.02$). Hepcidin was significantly inversely associated with non-heme iron absorption ($p = 0.04$), but had no significant impact on heme iron absorption in either pregnant or non-pregnant subjects.

Conclusion: Our study suggests that absorption of heme iron from animal based foods provides a highly bioavailable source of dietary iron during pregnancy that is not substantially affected by hepcidin concentrations or iron stores.

Introduction

Iron (Fe) deficiency is a public health problem affecting 1.62 billion people globally, with pregnant women being at increased risk (1). Anemia is associated with labor/delivery complications, preterm delivery, low birth weight, reduced infant Fe status, impaired mother-child interactions and increased infant and maternal mortality (2-4). Due to the adverse outcomes of maternal Fe deficiency, an understanding of dietary Fe bioavailability from all dietary iron sources is vital. These data will help inform the development of public health programs and nutritional recommendations. Dietary Fe is obtained from non-heme (mostly plant-based) and heme (mostly animal based) sources. While details of non-heme Fe absorption are largely known, mechanisms of heme Fe absorption remain elusive (5). In non-pregnant women, heme Fe absorption has been found to be 3-fold higher than that of non-heme Fe absorption (6). Non-heme Fe absorption has also been found to be more responsive to alternations in body Fe stores in comparison to heme Fe absorption in men (7). The impact of the increased Fe demands of pregnancy on heme versus non-heme Fe absorption remains unknown and to our knowledge heme Fe absorption has not been assessed during pregnancy.

Hepcidin is a small hormone produced by the liver that is now known to be a key regulator of Fe homeostasis (8). This hormone is known to be inversely associated with absorption of non-heme Fe in non-pregnant women and men (9;10) as discussed in Chapter 2. At present no human data on the role of this hormone on iron absorption during pregnancy is currently available. An improved understanding of the role of hepcidin concentration on absorption of dietary iron from all sources could potentially provide valuable information and therapeutic options for anemic pregnant women. Furthermore the degree to which heme Fe absorption is regulated by circulating hepcidin concentrations may provide additional insight into heme Fe homeostasis.

To address these issues we undertook an Fe absorption study using a dual stable isotope approach in pregnant and non-pregnant subjects. Iron absorption from an intrinsically labeled heme source was compared to labeled ferrous sulfate. We hypothesized women would have higher Fe absorption from the heme source, and that hepcidin would be associated with both heme and non-heme Fe absorption.

Subjects and Methods

Twenty non-smoking, pregnant women, ages 16-32 years, were recruited between 2008-2009 from the Rochester, NY Strong Midwifery Group and the Rochester Adolescent Maternity Program (RAMP). All women were healthy with uncomplicated pregnancies at the time of recruitment. Exclusion criteria at entry into the study included: gestational diabetes, hypertension, preeclampsia, underlying malabsorption diseases and diagnosed medical conditions known to impact Fe homeostasis. Informed written consent was obtained from each subject and the study was approved by the Institutional Review Board of Cornell University and the University of Rochester Research Subjects Review Board. The study was registered with clinicaltrials.gov as NCT01019096 (http://www.clinicaltrials.gov).

Twelve healthy non-smoking, non-pregnant women ages 18-27 years were recruited in 2009 from Ithaca, NY. Exclusion criteria included underlying malabsorption diseases or diagnosed medical conditions known to impact Fe homeostasis. Non-pregnant participants did not consume any supplements for one month prior to the study and for the duration of the study. Informed written consent was obtained from each subject and the study was approved by the Institutional Review Board of Cornell University.

Stable iron isotopes were purchased as the metal; ^{57}Fe at 94.69% enrichment and ^{58}Fe at 93.34% enrichment from Trace Sciences International (Ontario, Canada).

The non-heme Fe tracer (^{57}Fe) was prepared as ferrous sulfate as a sterile, pyrogen-free solution by Anazao Health (Tampa, FL). The Fe tracer (^{58}Fe) used for intrinsic labeling of heme was prepared as ferrous citrate in our laboratory and was sent to Analytical Research Labs (Oklahoma City, OK) for sterility testing. Isotopic composition of tracers was validated using Magnetic Sector Thermal Ionization Mass Spectrometry (TIMS) (Thermo Scientific Triton TI Thermo Fisher Scientific Inc, Bremen, Germany). The heme iron source was prepared by intrinsically labeling (^{58}Fe) porcine muscle and red blood cells (RBC) at Baylor College of Medicine following previously published methods, (11) as approved by the Baylor College of Medicine Institution Animal Care and Use Committee. In brief, a 3-day old female piglet was housed in a stainless steel stall and fed a diet to induce Fe deficiency (Fe-free sow replacement milk, Research Diets, Inc.). Piglets are typically given intramuscular Fe at birth (100-200 mg) to prevent Fe deficiency. The standard Fe injection was replaced with two 75 mg doses of ^{58}Fe, as ferrous citrate. At 28 days of age the piglet was sacrificed at a facility, Texas A&M University, approved by the USDA and the State of Texas. The RBCs and muscle (rich sources of heme iron) were collected, processed (11), and used as the intrinsically labeled heme Fe source for this study. Enrichment of ^{58}Fe in the muscle and RBCs was quantified by TIMS and found to be 18.0 ± 0.80% and 17.3 ± 0.01% (mean ± SD) respectively.

To accommodate the timing of subject recruitment two batches of the heme Fe meal were prepared. In the first batch prepared for the pregnant subjects, enriched ground pork (203 grams) and enriched RBCs (3.9 grams) were combined with tomato sauce (Hunts, Dallas, TX) and chili seasoning (McCormick, Sparks, MD). Based on preliminary results with the pregnant population, a slightly lower ^{58}Fe dose from the labeled pork meal was used for the non-pregnant subjects in order to dose a larger sample population and still achieve adequate enrichment values. Previous research has

found no significant difference in iron absorption from meat consumed alone versus meat consumed with additional hemoglobin (12). In the second batch of pork prepared for the non-pregnant subjects, non-enriched ground pork (198 grams) and enriched RBCs (3.5 grams) were combined with tomato sauce and chili seasoning. The chili was well mixed and frozen as individual 320 g servings until use. Aliquots of both heme meal batches were sent to Microbac Laboratories, Inc., (Warrendale, PA) for food safety testing and analyzed for total isotopic content by TIMS and total Fe content by atomic absorption spectrophotometry (AAS) (PerkinElmer Analyst 800; PerkinElmer Inc, Norwalk, CT).

Clinical studies in the pregnant participants were undertaken at Strong Memorial Hospital's Clinical and Translational Science Center (CTSC). Women came to the CTSC during the last trimester of pregnancy (gestation 32-35 weeks) on two consecutive days with each visit lasting approximately five hours. Subjects were asked to discontinue prenatal supplementation for 3 days prior to the study and two days following ingestion of each labeled test food. The morning of the first absorption study, each woman's height and weight were recorded with the use of a stadiometer and a calibrated scale. Fasted subjects received a standard breakfast (bagel, jelly/butter and non-ascorbic acid and non-Fe fortified apple juice) and 2 hours later ingested the Fe test meal (intrinsically labeled ^{58}Fe pork or ^{57}Fe as ferrous sulfate). The serving of intrinsically labeled pork provided 0.46 mg of ^{58}Fe from pork and 0.46 mg from RBCs for a total ^{58}Fe dose of 0.92 mg and 7.9 mg total Fe per serving. The ferrous sulfate administered was given as, 8.2 mg of ^{57}Fe as ferrous sulfate (total Fe, 8.6 mg) flavored with 2 mL of raspberry syrup containing 0.391% ascorbic acid (Humco, Texarkana, TX). Both forms of iron were fed to fasted subjects without other food or liquids. The two forms of Fe were fed randomly (coin toss for order) such that half of the women received the heme meal on the first day and the ferrous sulfate on the second day with

the other women consuming these forms of iron in the reverse order. Each subject served as their own control for comparing heme versus non-heme iron absorption. After each of the test meals were ingested, women remained in the CTSC for an additional two hours before ingesting a standard lunch (chicken noodle soup, apple, and pretzels). To minimize the influence of other dietary factors on Fe absorption all women were given a standard dinner (chicken, green beans, rice, carrots, ranch dressing, peaches and ice cream) and snacks to take home and consume. The pregnant adolescents were asked to stay overnight at the CTSC between test meals to minimize transportation difficulties. All subjects were asked to consume the same amounts and types of foods on the second test day as those ingested on the first day. Subjects returned to the CTSC two weeks after the second Fe tracer was ingested in order to collect a 15 mL venous blood sample.

Clinical studies in non-pregnant subjects were undertaken at the Human Metabolic Research Unit (HMRU) at Cornell University, using the same protocol as utilized for the pregnant subjects. The intrinsically labeled pork provided 0.6 mg of labeled ^{58}Fe RBCs and 9.9 mg of total Fe per serving. For the non-heme absorption study women ingested an 8.2 mg dose of ^{57}Fe as ferrous sulfate (total Fe, 8.6 mg) flavored with 2 mL of raspberry syrup (Humco, Texarkana, TX). Subjects remained in the HMRU for two hours and then were fed a standard lunch. As in the pregnant cohort, dinner and snacks were provided for subjects to take home. Subjects returned to the HMRU two weeks post-dosing for the blood draw.

Hemoglobin and hematocrit were analyzed in blood samples by the Strong Memorial Hospital's clinical laboratory using the Cell Dyn 4000 system (for pregnant subjects) and using a Coulter hematology analyzer at the HMRU (for non-pregnant subjects). Whole blood was centrifuged and serum was stored at -80°C until analysis. Serum ferritin was measured by a commercially available enzyme immunoassay procedure (Ramco Laboratories, Inc Stafford Texas). An indicator of inflammation

(C-reactive protein, CRP) was assessed using the Immulite®1000 immunoassay system (Tarrytown, NY). Serum soluble transferrin receptor (TfR) was measured with an enzyme linked immunosorbent assay (ELISA) (Ramco Laboratories, Inc Stafford Texas). Total body Fe (TBI) was calculated by the ratio of serum transferrin receptor to serum ferritin as described by Cook et al.; (total body Fe (mg/kg) = -[log (serum transferrin receptor/serum ferritin) – 2.8229]/0.1207) (13). Serum folate, vitamin B_{12} and erythropoietin were measured using the Immulite®1000 immunoassay system (Tarrytown, NY). Serum leptin was measured using a commercially available ELISA kit (Millipore, Billerica, MA). Serum samples were sent to Intrinsic Life Sciences for analysis of serum hepcidin using a competitive serum enzyme-linked immunosorbent assay (C-ELISA) (Intrinsic Life Sciences, La Jolla California).(14). The lower limit of detection for this assay is 5 μg/L.

To assess iron absorption, whole blood (0.5 mL) was digested and iron was extracted using anion exchange chromatography (15). All acid used was ultrapure (Ultrex, JT Baker). Iron isotope ratios ($^{57/56}$Fe, $^{58/56}$Fe and $^{54/56}$Fe) were measured using TIMS and Fe absorption was determined as described in previous publications (15-17). The enrichment of each isotope in RBCs was measured as the degree to which the natural abundance ratio in RBC was increased as a result of absorption of the stable Fe tracer using the formula; [($^{57/56}$Fe ratio in sample - $^{57/56}$Fe natural abundance ratio)/ $^{57/56}$Fe natural abundance ratio] *100. A similar equation was used to assess the enrichment of ^{58}Fe in RBC from the labeled heme meal. Natural abundance (NA) Fe isotopic ratios utilized were 0.02317 for $^{57/56}$Fe and 0.00307 for $^{58/56}$Fe. The total circulating Fe (mg) was estimated using the subjects' weight (kg), a blood volume estimate (65 mL/kg for non-pregnant and 70 mL/kg for pregnant subjects), hemoglobin concentration (kg/L) and the concentration of Fe in hemoglobin (3.47 g/kg) as reported by Fomon et al.(18). Total Fe incorporated was then calculated

using the RBC enrichment, total circulating Fe, the Fe dose administered and the natural abundance of isotope given (NA_{57}, 0.0214; NA_{58}, 0.00287). Final values for Fe absorption were determined based on the assumption that 80% of the absorbed Fe would be incorporated into erythrocytes (19;20). An additional correction factor was used in the Fe absorption calculations in order to correct for the small amount of ^{58}Fe that was contributed by the ^{57}Fe tracer (16).

Data were analyzed using JMP 8.0 (SAS Institute INC, Cary, NC). Linear regression analyses were used to examine relationships between Fe status, hepcidin and Fe absorption. Paired t-tests were used to compare the differences of relationships between heme and non-heme Fe absorption within subject groups. Differences between subject groups were compared using a t-test or the Wilcoxon rank sum test for nonparametric data. Serum hepcidin concentrations below the limit of detection were assigned a value of 2.5 μg/L for data analysis (a value halfway between zero and the limit of detection, 5 μg/L). A sample size of 20 pregnant women was selected to provide a power of 80% using an alpha level of 0.05 to detect a 4.5% difference in iron absorption, allowing for an attrition rate of 20%. Variables were tested for normality using goodness-of-fit test and those that were not normally distributed were transformed (natural log) prior to statistical testing. Results were analyzed with and without the two subjects who developed preeclampsia (PE). The results and p-vales are given for the complete sample size unless inclusion of PE subjects impacted the significance as noted in the results. Differences were considered significant if $p < 0.05$**.**

Results

General characteristics of the study subjects are shown in **Table 3.1**.

Table 3.1

General characteristics of the participants[1,2]

Variable	Pregnant (n = 18)	Non-Pregnant (n = 11)
Age at enrollment (y)	18.5 ± 4.0 (16 - 32) [a]	21.5 ± 2.4 (18 - 27) [b]
BMI (kg/m^2) at time of study	32.5 ± 6.9 (23.3 - 46.5) [a]	23.0 ± 2.2 (18.9 - 25.8) [b]
Pre-pregnancy BMI (kg/m^2)	25.0 ± 7.1 (20.7 - 43.6)	N/A
Gestational Age at Study (wks)	33.4 ± 0.9 (32.0 - 34.6)	N/A
Race		
African American	50%	0%
Caucasian	50%	73%
Native American	0%	9%
Asian	0%	18%
Ethnicity		
Hispanic	39%	0%
Non-Hispanic	61%	100%

[1]All normally distributed values are mean ± SD and all non normally distributed values are median ± SD (range in parentheses)

[2]Variables that are significantly different among pregnant and non-pregnant subjects are denoted with a different letter ($p < 0.05$)

Of the pregnant subjects enrolled in the study; those under the age of 18 y were classified as adolescents (50%) and the remaining 50% were ≥ 19 yrs and were classified as adults. Among the pregnant women enrolled, 50% entered pregnancy with a normal BMI, 22% were overweight (BMI > 25 kg/m^2) and 28% were obese (BMI > 30 kg/m^2) prior to pregnancy. None of the pregnant or non-pregnant subjects were underweight. Three of the non-pregnant subjects were classified as overweight and the remaining nine subjects had a normal BMI.

Three subjects did not complete the study. One pregnant subject did not consume all of the pork meal and was withdrawn from the study. Another pregnant adolescent completed the two day study but did not attend the 2-week blood draw appointment. One non-pregnant subject did not attend the second day of dosing and was withdrawn from the study. The final study population included 18 pregnant and 11 non-pregnant subjects. Two pregnant study participants (ages 16 and 20) developed preeclampsia (PE). In these two women, blood samples were obtained at delivery which occurred at 10 days post-dosing in one subject and on day 14 post-dosing in the second subject. Iron status indicators are described in **Table 3.2**. Among the pregnant subjects, 61% had depleted Fe stores (serum ferritin < 20 μg/L) and 22% had negative TBI values. The majority (67%) of the pregnant women had non-detectable serum hepcidin levels in the third trimester of pregnancy and these subjects were also the women with depleted iron stores. All but one of the non-pregnant women were Fe replete, with one having mild anemia (Hb = 118 g/L). Only three non-pregnant subjects had non-detectable levels of serum hepcidin and they likewise all had serum ferritin values near depletion. None of the pregnant subjects had folate (< 6.8 nmol/L) or B-12 deficiency (< 148 pmol/L) (21). All non-pregnant subjects had normal folate levels; however one subject had a low B-12 value (111 pmol/L) but was not anemic (Hb = 137 g/L). The two subjects with PE had elevated CRP (66.1, 6.1 mg/L).

Table 3.2

Iron status indicators for pregnant and non-pregnant subjects[1,2]

Variable	Pregnant (n = 18)	Non-Pregnant (n = 11)
Hemoglobin (g/L)	114 ± 9 [a]	130 ± 9 [b]
(% Anemic)	< 110 g/L: 39%	< 120 g/L: 9%
Serum ferritin (μg/L)	17.5 ± 16.4 [a]	40.5 ± 23.3 [b]
< 12 μg/L (% Fe Deficiency)	22%	0%
< 20 μg/L (% Fe Depletion)	61%	9%
Serum transferrin receptor (mg/L)	6.1 ± 1.5 [a]	4.2 ± 1 [b]
> 8.5 mg/L	11%	0%
Total Body Iron (mg/kg)	2.3 ± 3.0 [a]	6.0 ± 1.7 [b]
< 0 mg/kg	22%	0%
Serum hepcidin (μg/L)	2.5 ± 47.8 [a]	16.7 ± 13.8 [b]
C-reactive protein (g/L)	3.5 ± 15.1 [a]	0.66 ± 1.4 [b]
Folate (nmol/L)	37.9 ± 21.2 [a]	38.6 ± 6.7 [a]
Vitamin B-12 (pmol/L)	213.8 ± 113 [a]	223.6 ± 169.1 [a]
Erythropoietin (IU/L)	30.5 ± 18.0 [a]	12.7 ± 8.7 [b]
Leptin (μg/L)	40.6 ± 24.5 [a]	9.8 ± 6.1 [b]

[1]All normally distributed values are mean ± SD and all non normally distributed values are median ± SD

[2]Variables that are significantly different among pregnant and non-pregnant subjects are denoted with a different letter ($p < 0.05$)

Pregnant women absorbed a significantly greater percentage of Fe from the intrinsically labeled pork meal (47.7 ± 14.4%; p = 0.04) in comparison to absorption from a similar iron load ingested as ferrous sulfate (40.4 ± 13.2%), **Figure 3.1**.

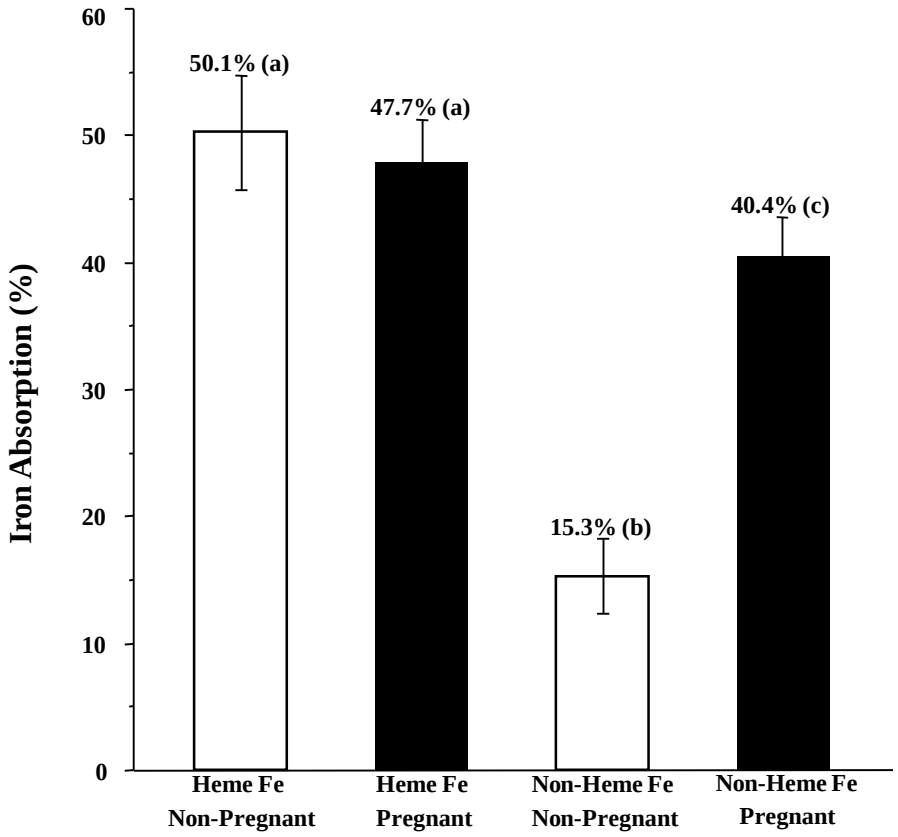

Figure 3.1 Heme and Non-Heme Iron Absorption.

Heme (intrinsically labeled ^{58}Fe pork) and non-heme (^{57}Fe as ferrous sulfate) iron absorption was examined in 18 pregnant and 11 non-pregnant subjects. Pregnant and non-pregnant subjects had significantly greater heme iron absorption compared to non-heme iron absorption ($p = 0.04$, $p < 0.0001$). Means that are significantly different from each other are denoted with a different letter ($p < 0.05$).

A similar pattern was observed among the non-pregnant women with Fe absorption from the intrinsically labeled pork meal (50.1 ± 14.8%) being significantly greater than absorption of ferrous sulfate (15.3 ± 9.7%, $p < 0.001$). Pregnant women absorbed significantly more non-heme Fe than non-pregnant women ($p < 0.001$), but no significant differences in heme Fe absorption were evident between the pregnant and non-pregnant women**.** The relative difference in heme versus non-heme Fe absorption was significantly lower in the pregnant women compared to the mean difference observed among the non-pregnant women (7.4 ± 14% vs. 35 ± 14%, $p<0.001$). Mean Fe absorption was not significantly affected by the order in which the two forms of Fe were fed. Estimations used for blood volume and RBC incorporation may impact the absolute values of absorption obtained. To minimize this possibility while assessing differences between groups the ratio of absorbed heme iron to non-heme iron (^{58}Fe absorption/^{57}Fe absorption) was compared between the pregnant (1.26 ± 0.44) and non-pregnant (4.8 ± 3.1) subjects and was also found to be significantly different ($p = 0.003$).

In pregnant subjects non-heme Fe absorption was inversely associated with TBI ($p = 0.02$, $r^2 = 0.29$) and hemoglobin ($p = 0.04$ $r^2 = 0.24$) and directly associated with TfR ($p = 0.003$ $r^2 = 0.43$). When the two PE patients were excluded the relationship between non-heme Fe absorption and hemoglobin lost significance. In contrast, relationships between heme Fe absorption and maternal TBI ($p = 0.08$) and TfR ($p = 0.06$) during pregnancy only approached significance. In non-pregnant subjects neither non-heme nor heme Fe absorption were significantly related to Fe status indicators with the exception of TfR which was significantly related to heme Fe absorption ($p = 0.04$, $r^2 = 0.39$). During pregnancy heme iron absorption was inversely associated with age ($p = 0.02$, $r^2 = 0.29$) with pregnant adolescents (≤ 18 y) absorbing significantly more heme Fe than adult women ($p < 0.001$). Absorption of ferrous

sulfate did not significantly differ between pregnant adolescents and adults. Age was not associated with serum hepcidin or measures of iron status and there were no significant differences in Fe status, serum hepcidin, parity, race/ethnicity, gestational age or BMI between the pregnant adolescents and adults in the study. Neither heme nor non-heme iron absorption were related to BMI, leptin or CRP. Among the pregnant subjects, CRP was directly related to serum hepcidin ($p = 0.005$), however, the relationship was no longer significant if the two subjects with preeclampsia were excluded. Likewise, serum hepcidin was not significantly associated with BMI or leptin.

As expected, serum hepcidin was related to iron status. In pregnant women hepcidin was directly associated with serum ferritin ($p< 0.001$, $r^2= 0.61$) and TBI ($p< 0.001$ $r^2= 0.51$). Similar but less significant relationships were noted in the non-pregnant subjects with serum hepcidin being significantly related to serum ferritin ($p = 0.03$ $r^2= 0.43$) and TBI ($p = 0.03$ $r^2= 0.44$).

Hepcidin was also significantly correlated with non-heme Fe absorption ($p = 0.04$, $r^2= 0.14$) when data from the pregnant and non-pregnant subjects were combined ($n = 29$). When the two subjects with PE were excluded from the analysis this relationship was further strengthened ($p = 0.009$, $r^2= 0.24$). Pregnant and non-pregnant subjects with non-detectable (<5 μg/L) serum hepcidin levels had significantly greater non-heme Fe absorption compared to subjects with detectable serum hepcidin ($p = 0.02$, $n = 29$). In contrast, heme Fe absorption was not significantly associated with serum hepcidin in the group as a whole or within the non-pregnant or pregnant cohorts. Moreover, no significant differences in heme Fe absorption were evident between those with detectable versus non-detectable serum hepcidin levels.

Discussion

Both pregnant and non-pregnant women absorbed a significantly higher fraction of dietary iron from an animal-based heme source compared to Fe absorption from ferrous sulfate. During pregnancy daily absorbed iron requirements increase ~4-fold presumably to support gestational Fe losses to the fetus, placenta and post-partum blood loss (22). During pregnancy the dietary recommendations for iron increase by ~9 mg/d (to 27 mg iron/day), which is typically met by supplemental iron. According to US data 36% of women are non-compliant with prenatal iron supplementation recommendations and use is often lowest in those at highest risk for anemia (low-income, black women) (23;24). In our study iron absorption was assessed from ~9 mg of ferrous sulfate compared to a similar Fe dose from meat. Our data indicated a typical animal-based meal containing ~9 mg of iron could provide ~95% of the daily absorbed iron requirement during pregnancy versus 81% which would be obtained from ~9 mg of ferrous sulfate given alone without a meal to fasted women (assuming an absorbed daily iron intake requirement of 4.5 mg). The non-heme dose was given under optimal absorption conditions (fasted, low-dose liquid with ascorbic acid) and when taken as part of a meal absorption would likely decrease. Prenatal supplements typically contain 30 - 60 mg of iron, and in our previous research pregnant women absorbed ~ 9.9 % from a 60 mg Fe supplement, which would exceed average iron requirements if taken on a regular basis (17).

The relative difference in absorption between the two Fe sources was significantly different between the pregnant and non-pregnant women. Among the non-pregnant women there was a 3.3 fold difference in Fe absorption from intrinsically-labeled pork versus ferrous sulfate compared to only a 1.2 fold increase during pregnancy. Similar differences in upregulation of heme iron absorption were evident when results were expressed as the ratio of heme to non-heme Fe absorption.

The lower difference in heme versus non-heme iron absorption observed among pregnant women was likely a consequence of their reduced iron stores. Lower iron stores were associated with significantly lower serum hepcidin among pregnant women. Down-regulation of hepcidin allows for an increased efflux of non-heme iron across the enterocyte. Although mechanisms of heme absorption remain uncharacterized, iron stores are thought to have less impact on heme iron absorption than on non-heme Fe absorption (7). A similar attenuated relationship between iron stores and heme Fe absorption was reported among blood donors (lower Fe status) in whom a 2-fold difference between heme and non-heme iron absorption was noted compared to the 5-fold difference in Fe-replete non-blood donors (7). The marked differences in iron status among our pregnant and non-pregnant subjects preclude us from making conclusions on the impact of pregnancy alone on heme and non-heme iron absorption. Additional research is needed to further understand the role of pregnancy, independent of iron status, on upregulating iron absorption. Not surprisingly given the fact that teen pregnancy disproportionately impact minorities. Although race was differed between the pregnant and non-pregnant cohort – at present there are no race-specific differences for iron deficiency nor is there any data to suggest cellular mechanisms of iron transport vary as a function of race.

The role of hepcidin in the regulation of Fe absorption during pregnancy has been largely unexplored. In the current study serum hepcidin was inversely associated with markers of Fe status in pregnant and non-pregnant populations. Likewise, previous research has found relationships between urinary hepcidin and maternal iron status (25). In our study non-heme Fe absorption was inversely related to serum hepcidin; women with non-detectable levels of serum hepcidin had significantly greater non-heme Fe absorption. Serum hepcidin explained ~24% of the variation in non-heme Fe absorption in healthy pregnant and non-pregnant subjects, which is

similar to our previous research in non-pregnant women (26%) (9). Likewise animal data have found hepatic hepcidin expression to decrease during pregnancy resulting in increased Fe absorption (26). Hepcidin regulates non-heme Fe absorption by binding to ferroportin, the sole known non-heme Fe exporter in the enterocyte, effectively blocking Fe flux (27). Hepcidin may also inhibit apical iron uptake in the enterocyte (28). The process by which heme exits the enterocyte has not been definitively characterized; it is not known if all of heme is degraded within the cell such that it is exported entirely using non-heme iron transport mechanisms or if some heme iron exits intact (29). These differences in iron metabolism may explain the lack of association between heme Fe absorption and hepcidin.

Limitations of the current study design include our estimation of RBC Fe incorporation. Assessment of this value in each subject would have required an infusion of a third stable Fe isotope and would have impacted our ability to adjust data obtained for isotopic fractionation. We chose instead to focus on the comparison in relative differences in bioavailability of the two oral forms of Fe ingested. With this approach each woman was their own control and differences in RBC Fe incorporation would not impact the relative absorption value. Because an intravenous tracer was not administered we estimated the fraction of absorbed Fe that would be incorporated into RBCs. In non-pregnant subjects this is relatively stable and typically averages 80% and we chose to use the same value for pregnant subjects. However, estimates of RBC Fe incorporation during pregnancy can be highly variable and we have previously found this to be affected by Fe supplementation and maternal serum ferritin (17). Other researchers have found values of RBC Fe incorporation as low as 70% (20) or 65% (30) during pregnancy. We re-fit our data using the reported estimate of 65% in place of 80% and found that this would increase non-heme Fe absorption from 40.4% to 49.8% and heme Fe absorption from 47.7% to 58.7% but did not change any of the

significant relationships reported. Furthermore, incorporation of dietary iron into maternal RBCs during pregnancy reflects maternal utilization of iron but does not capture the amount of iron that was transferred directly to the fetus. Early radiotracer studies demonstrated that placental transfer of orally ingested iron occurs within minutes of injection (31) or within hours of ingestion (32). In a prior study of non-heme iron absorption we found that the amount of non-heme dietary Fe transferred to the fetus underestimated maternal Fe absorption by approximately 5% (33).

In summary, heme iron absorption was not influenced by maternal iron stores or serum hepcidin in either the pregnant or non-pregnant women. Absorption of heme iron was significantly greater than absorption of a similar load of non-heme, supplemental iron. Compliance with prenatal iron supplementation is often lowest in those with the greatest risk of anemia thus emphasis on how to best meet iron requirements from the diet alone is an important concern. The significantly greater bioavailability of heme iron may help achieve dietary iron demands during pregnancy. Further research is required to understand the mechanisms by which heme iron absorption is enhanced and to characterize the ability of these two forms of iron to provide for fetal iron demand.

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CHAPTER 4

MATERNAL SERUM HEPCIDIN IMPACTS HEME AND NON-HEME PLACENTAL IRON TRANSFER DURING PREGNANCY ‡

‡ **Melissa F. Young, Ian Griffin, Eva Pressman, Allison W. McIntyre, Elizabeth Cooper, Thomas McNanley, Z. Leah Harris, Mark Westerman, Kimberly O. O'Brien.** **Maternal serum hepcidin impacts heme and non-heme placental iron transfer during pregnancy. Submitted to American Journal of Clinical Nutrition.**

Abstract

Background: Determinants of placental transport of dietary iron (Fe) remain largely uncharacterized.

Objective: To elucidate the determinants of heme and non-heme Fe transfer to the neonate.

Design: A sample of 19 pregnant females (age 16-32 y) ingested intrinsically labeled ^{58}Fe-heme and a non-heme iron source (^{57}Fe as ferrous sulfate) during the third trimester of pregnancy. At delivery maternal and cord blood samples were obtained to assess Fe transfer to the neonate as a function of maternal/neonatal Fe status (serum ferritin (SF), transferrin receptor (TfR), hemoglobin (Hb), total body iron (TBI) and serum hepcidin).

Results: There was a significantly greater enrichment of ^{58}Fe-heme tracer in cord blood compared to ^{57}Fe-non-heme tracer enrichment ($p = 0.02$) and a greater fraction of the maternally absorbed heme Fe was transferred to the neonate ($p < 0.0001$). Net non-heme Fe (mg) transferred to the neonate was related to maternal SF ($p = 0.0008$), Hb ($p = 0.005$), TBI ($p < 0.0001$), TfR ($p = 0.0002$) and serum hepcidin ($p = 0.002$) at delivery. Likewise, net heme Fe (mg) transferred was associated with maternal SF ($p = 0.003$), Hb ($p = 0.02$), TBI ($p = 0.001$), TfR ($p = 0.01$) and serum hepcidin ($p = 0.004$). Iron transfer of both heme and non-heme iron was also associated with neonatal Hb ($p = 0.008$, $p = 0.004$). Mothers with non-detectable serum hepcidin transferred significantly more non-heme ($p = 0.003$) and heme ($p = 0.002$) Fe to their neonates than subjects with detectable levels.

Conclusion: There appears to be preferential fetal utilization of maternally ingested heme Fe compared to non-heme Fe during pregnancy. Maternal serum hepcidin and maternal/neonatal Fe status play a role in regulating heme and non-heme iron transfer.

Introduction

Neonatal iron (Fe) endowment at birth has increasingly been recognized as an important factor in subsequent health outcomes (1). During the third trimester of pregnancy the fetus obtains the majority of its Fe stores and suboptimal placental Fe transfer may set the stage for increased risk of postnatal Fe insufficiency. The importance of maternal Fe status in the establishment of the neonatal Fe endowment at birth has been controversial. Previously, the fetus was believed to be a "perfect parasite" capable of extracting all necessary Fe from the mother regardless of her Fe stores. However, there are now growing data to support a relationship between maternal anemia and suboptimal neonatal Fe status at birth (2-5). Moreover, there is a growing recognition of the long-term and irreversible effects of neonatal anemia on cognitive, motor and social-emotional outcomes (6-8). We previously reported a relationship between maternal Fe status and transfer of non-heme Fe to the fetus (9;10). However, the regulation of heme Fe metabolism during pregnancy remains largely unexplored. Hepcidin is a systemic regulator of Fe homeostasis and functions by binding to ferroportin and causing it to be internalized, thereby blocking Fe export from the enterocyte and Fe release from macrophages and the liver (11). At this time there are limited human data on the role of hepcidin in the regulation of placental heme or non-heme Fe transport, although animal data has suggested a link between hepcidin and non-heme Fe homeostasis during pregnancy (12;13). The goal of this study was to evaluate placental Fe transfer of both dietary heme and non-heme Fe during the third trimester of pregnancy in relation to maternal and neonatal Fe status and serum hepcidin.

Subjects and Methods

Twenty pregnant study volunteers, age 16-32 years, were recruited from the Strong Midwifery Group and the Rochester Adolescent Maternity Program (RAMP) in Rochester, NY. Only healthy non-smoking females with uncomplicated pregnancies were asked to participate. Women and adolescents were excluded if they had gestational diabetes, hypertension, preeclampsia, underlying malabsorption diseases or medical problems known to impact Fe homeostasis at the time of enrollment. The study was approved by the Institutional Review Board of Cornell University and the University of Rochester Research Subjects Review Board and informed written consent was obtained from all subjects. The study was registered with clinicaltrials.gov as NCT01019096 (http://www.clinicaltrials.gov). Data on Fe absorption in these study participants was discussed in Chapter 3.

Isotope Preparation

Iron isotopes were purchased from Trace Sciences International (Ontario, Canada) as the metal (^{57}Fe at 94.69% enrichment and ^{58}Fe at 93.34% enrichment). The non-heme Fe tracer (^{57}Fe) was converted into ferrous sulfate solution by Anazao Health (Tampa, FL). The Fe tracer used to intrinsically label heme (^{58}Fe) was converted into ferrous citrate and then analyzed for sterility by Analytical Research Labs (Oklahoma City, OK). Iron isotopic composition of the final tracer solutions tracers were assessed using a Thermo Scientific Triton TI Magnetic Sector Thermal Ionization Mass Spectrometer (TIMS) (Thermo Fisher Scientific Inc, Bremen, Germany). Intrinsic heme labeling was undertaken at Baylor College of Medicine using a protocol approved by their Animal Use Committee. Methods were adapted from a previous study by Etcheverry et al. (14). Briefly, intrinsic labeling of the porcine muscle and red blood cells (RBC) Fe was accomplished by two intramuscular 75 mg doses of ^{58}Fe into a 3-

day old female piglet. The piglet was sacrificed after 28 days at a USDA and State of Texas approved facility at Texas A&M University. Red blood cells, a rich source of Hb, and muscle, a rich source of myoglobin, were collected and used as the labeled heme Fe source for the study. The muscle (^{58}Fe enrichment of 18.0 ± 0.80%) and red blood cells (^{58}Fe enrichment of 17.3 ± 0.01%) were analyzed separately for enrichment of ^{58}Fe by TIMS as previously reported in Chapter 3.

Study design and isotope dosing

Subjects participated in a 2-day Fe absorption study during the third trimester of pregnancy (week 32-35 of gestation) at Strong Memorial Hospital's Clinical and Translational Science Center (CTSC). All subjects discontinued any Fe or prenatal supplementation for three days prior to the first day of Fe dosing. Fasted participants were asked to come to the CTSC on two consecutive days. Each individual's height and weight were taken with the use of a stadiometer and a calibrated scale on the morning of the first study day. On each test day a standard breakfast was provided and each research participant then remained in the CTSC for two hours before receiving each Fe test meal. All participants consumed both a heme (^{58}Fe intrinsically labeled ground pork) and non-heme (^{57}Fe as ferrous sulfate) test meal in a random order. The heme Fe dose included approximately 203 grams of enriched ground pork and 3.9 grams of enriched RBCs cooked into a chili mix with tomato sauce (Hunts, Dallas, TX) and chili seasoning (McCormick, Sparks, MD). The net Fe load ingested from the heme and non-heme test meals were similar with each containing approximately 8 mg of iron. The heme meal provided 0.46 mg of labeled ^{58}Fe from intrinsically labeled pork muscle and 0.46 mg from intrinsically labeled RBCs for a total dose of 0.92 mg ^{58}Fe (total Fe, 7.9 mg). The non-heme Fe dose consisted of an oral dose of 8.2 mg of ^{57}Fe as ferrous sulfate (total Fe, 8.6 mg) flavored with 2 mL of raspberry syrup

containing ascorbic acid (Humco, Texarkana, TX) without food. The total Fe content of the heme meal and non-heme Fe dose was validated using atomic absorption spectrophotometry (AAS) (PerkinElmer Analyst 800; PerkinElmer Inc, Norwalk, CT). Pregnant women and adolescents remained in the CTSC for two hours after ingesting each test meal before being fed a standard lunch. Standard dinner and snacks were provided for the subjects to take home and consume. Pregnant adolescents remained as inpatients overnight at the CTSC to avoid transportation difficulties and to insure food intake was controlled. On the second test day, the same foods in the same amounts were consumed as those ingested on the first day.

All study participants were followed until delivery and their medical charts were flagged to insure delivery samples would be obtained. At admission a 15 mL maternal blood sample was obtained and at delivery a 30 mL cord blood sample was taken.

Laboratory Analysis

Whole blood samples collected at delivery were transported to Strong Memorial Hospital's clinical laboratory for analysis of hemoglobin (Hb) using the Cell Dyn 4000 system. Hemoglobin measures were also obtained at bedside using a HemoCue (Lake Forest, CA). When clinical laboratory values for neonatal hemoglobin were not available (due to logistical and technical problems with sample clotting, etc.) the HemoCue values were utilized. In other whole blood samples, serum was separated on site and shipped to Cornell on dry ice for analysis of Fe status indicators. Serum ferritin (SF) and serum soluble transferrin receptor (TfR) were measured with commercially available kits from Ramco Laboratories, Inc. (Stafford Texas).

From these two markers, total body Fe was calculated as described by Cook et al.; (total body iron, TBI, (mg/kg) = -[log (serum transferrin receptor/serum ferritin) – 2.8229]/0.1207) (15). C-reactive protein (CRP), serum folate, vitamin B_{12} and erythropoietin (EPO) were measured using the Immulite®1000 immunoassay system (Tarrytown, NY). Intrinsic Life Sciences measured maternal and neonatal serum hepcidin using a competitive serum enzyme-linked immunosorbent assay (C-ELISA) specific for the mature peptide (Intrinsic Life Sciences, La Jolla California) (16). The lower limit of detection for this assay is 5 µg/L (16).

Isolation of iron from samples/mass spectrometry

Samples of whole blood (0.5 mL) were digested with 2 mL of nitric acid and 0.1 mL hydrogen peroxide by heating in glass flasks on a hot plate. Digested samples were evaporated to dryness and reconstituted in 2 mL of ultrapure 6M hydrochloric acid (Ultrex, JT Baker). Iron was extracted from the digested blood samples using anion exchange chromatography. The eluate was dried on a hot plate and then reconstituted in 40 µL 3% nitric acid. Extracted Fe samples (8 µL), silica gel (3 µL) (Sigma-Aldrich, Inc., St. Louis, MO) and 0.7 N phosphoric acid (3 µL) were loaded onto degassed ultrapure zone-refined rhenium filaments (H. Cross Company Weehawken, NY). Iron isotope ratios ($^{57/56}$Fe , $^{58/56}$Fe and $^{54/56}$Fe) were measured using TIMS (9). All acids used were ultrapure (Ultrex JT BAKER, Phillpsburg, NJ).

Calculation of iron transfer to neonate

As denoted in **Figure 4.1**, the oral iron tracer administered to the pregnant women has two primary fates once it crosses the enterocyte, it is either incorporated into the maternal RBC or used for maternal stores or it is transported across the placenta to be incorporated into the neonate's RBC or used for neonatal iron stores.

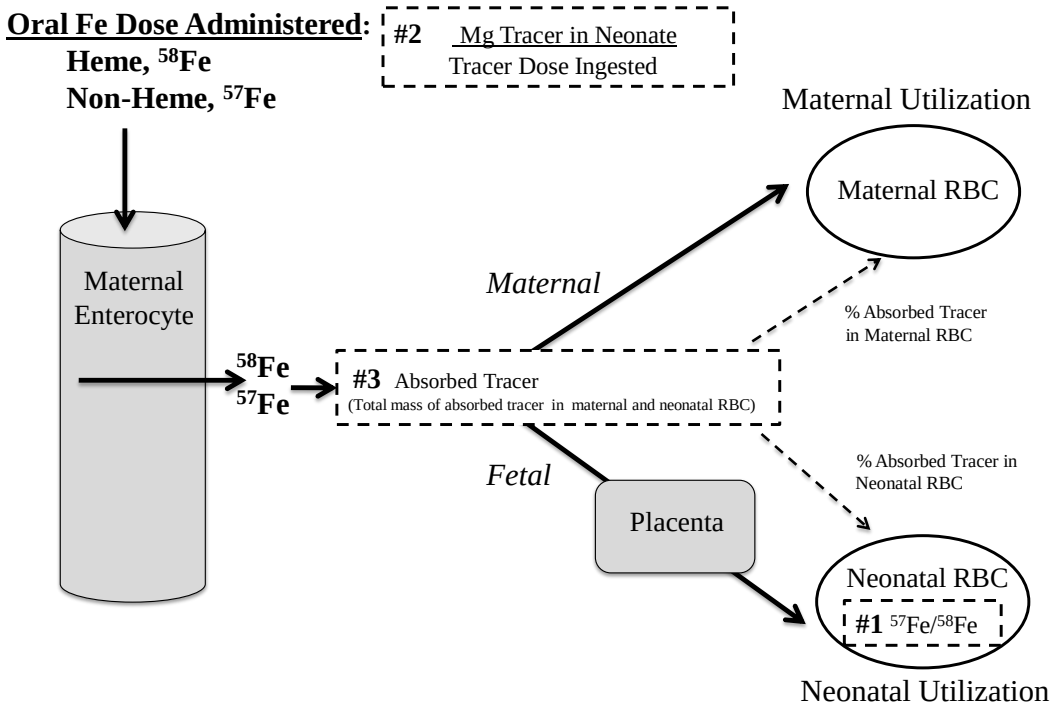


Figure 4.1 Iron Partitioning During Pregnancy

Partitioning of the two oral iron stable isotopes (^{57}Fe and ^{58}Fe) administered to pregnant women in the third trimester of pregnancy was assessed by three primary methods. The first method assessed the dose adjusted delta percent enrichment of each isotope in the neonatal RBC. The second method calculated the net milligrams of tracer present in the neonatal RBC mass divided by the net tracer ingested by the mother. The final approach assessed the net amount of iron isotope present in maternal and neonatal RBC then looked at the relative partitioning of absorbed isotope between the mother and the neonate as a fraction of the total isotope recovered in RBC's.

As discussed in Chapter 3, measures of iron absorption during pregnancy should more accurately be described as maternal utilization. Isotope enrichment in maternal RBC underestimates net maternal absorption because a fraction of the absorbed iron is directly transferred to the neonate. Previous research has indicated that approximately 7.5% of the absorbed non-heme iron tracer is found in the neonate at birth when compared to the circulating mass of iron present in the maternal RBC (10). Iron transfer to the fetus is also known to be rapid and is not significantly related to the time between dosing and delivery, indicating that there is negligible transfer of the tracer once incorporated in the mother's RBC (10;17;18). Thus isotopic enrichment in the maternal and cord blood samples gives a good depiction of maternal iron partitioning during late pregnancy.

In our study placental iron transfer was assessed in multiple ways as described below. Each step attempts to more accurately characterize the net amount of iron transferred to the neonate but each step is also dependent on additional assumptions of neonatal/maternal blood volume and fraction of iron incorporated into the neonatal/maternal RBC and therefore may be introducing more error into the estimates. It is important to note that while this may impact the estimate of the absolute quantity of iron incorporated it should not impact relative differences observed between heme and non-heme iron transfer within a subject as the assumptions utilized are identical for both tracers.

Approach 1

For the first approach utilized; enrichment of ^{57}Fe and ^{58}Fe in cord blood was directly analyzed. The delta percent enrichment of each isotope was measured as the degree to which the natural abundance ratio was increased as a result of red blood cell incorporation of the stable Fe tracer as previously reported (10) using the formula:

#1) Δ % enrichment ^{57}Fe =

$$\frac{(^{57/56}\text{Fe ratio in sample} - {}^{57/56}\text{Fe natural abundance ratio})}{{}^{57/56}\text{Fe natural abundance ratio}} * 100$$

The same equation was utilized for the delta percent enrichment of ^{58}Fe from the labeled heme meal. The natural abundance Fe isotopic ratios utilized were 0.02317 for $^{57/56}$Fe and 0.00307 for $^{58/56}$Fe.

Doses of each isotope were selected to achieve the same relative enrichment in blood assuming that each tracer dose was absorbed to the same extent (i.e. a dose of 10 mg ^{57}Fe would give the same enrichment as a dose of 1.34 mg of ^{58}Fe given the differences in the natural abundance of each tracer (natural abundance (NA) values utilized were 0.0214 for NA_{57} and 0.00287 for NA_{58}). Subtle differences in net dose administered occurred between subjects. To control for this variability, all measures used a dose adjusted delta % enrichment for both the ^{57}Fe and ^{58}Fe. For this measure the observed delta percent excess in cord blood and actual dose administered was used to obtain an expected delta percent excess using the mean tracer dose of ^{57}Fe administered (8.2 mg) and a corresponding mean dose of ^{58}Fe of 1.1 mg.

Approach 2

To further characterize partitioning of iron to the fetus the amount of iron in the neonate was examined as a fraction of iron tracer dose given to the mother (**Figure 4.1**). To obtain this measure the neonatal mass of circulating iron was first estimated assuming a neonatal blood volume of 80 mL per kilogram (19;20) and an Fe content of hemoglobin of 3.47 g/kg (21;22) with the following formula:

Total circulating neonatal iron (Fe_{circ}) =

Blood volume (80 mL/kg) * Hb (kg/L) * Wt (kg) * [Fe] of Hb (3.47 g/kg)

The net mg quantity of Fe isotope present in the infant circulation was then calculated for both the heme and non-heme Fe as;

Net non-heme iron transfer (mg) = **(EQN 1)**

(Dose adjusted Δ % enrichment $^{57}Fe/100)$* (Fe_{circ})* (NA_{57})

The same equation was utilized for the ^{58}Fe tracer substituting in NA_{58} and the dose adjusted delta % enrichment of ^{58}Fe to calculate the net heme Fe transfer. Additional calculations were undertaken to account for the small amount of ^{58}Fe that was present and absorbed from the ^{57}Fe tracer, as the ^{57}Fe tracer contained 2.1% of ^{58}Fe.

#2) Percent of Maternal Ingested Non-heme Fe Tracer Transferred to Neonate = (Net non-heme Fe transfer- EQN 1 / Mean maternal ^{57}Fe dose (mg) administered)*100

A similar equation was used to calculate the net heme Fe transfer as a fraction of the corrected mean dose of ^{58}Fe administered to the mother (percent of maternal ingested heme Fe tracer transferred to neonate). This approach uses estimates of neonatal blood volume and circulating neonatal iron mass.

Approach 3

Finally the relative partitioning of net absorbed iron tracer was examined in relation to the total quantity of dose recovered in the maternal and neonatal RBCs. The total mg recovered in the mother was calculated with the following formula; % Non-heme Fe absorption *0.01 * ^{57}Fe dose/ RBC incorporation. Equation 1 above details

the net mg recovered in the neonate. The total absorbed dose was calculated as the sum of these two net quantities.

Total Absorbed Tracer Dose =

mg recovered in neonate (EQN-1) + mg recovered in mother

Then the relative partitioning of tracer to the neonate could be calculated by the following formula:

#3) % of Maternal Absorbed Non-Heme Fe Tracer Transferred to Neonate =

$$\frac{\text{(Net non-heme Fe transfer- EQN 1)} * (100)}{\text{Total Absorbed Dose}}$$

A similar equation was utilized for heme Fe to calculate the percent of maternal heme dose absorbed and transferred to the neonate (percent of maternal absorbed heme Fe tracer transferred to neonate). This equation has the advantage of taking into account differences in iron absorption and examining relative partitioning of the isotope; however, estimates on maternal RBC incorporation and neonatal/ maternal blood volume and circulating iron mass are utilized in the calculations.

Data analysis

All statistical analyses were completed using JMP 8.0 (SAS Institute INC, Cary, NC). Variables that were not normally distributed were transformed prior to statistical testing (using the natural log). Linear regression analyses were used to examine the relationships between Fe status, hepcidin and placental transfer. Multiple linear regression analyses using forward stepwise regression were utilized to identify significant determinants of heme and non-heme placental Fe transfer. The significance of relationships between heme and non-heme Fe transfer and Fe status within subject

groups was assessed using Paired t-tests. Non-detectable serum hepcidin levels (< 5 ng/mL) were given a value of 2.5 ng/mL for data analysis purposes, as reported in Chapter 3. Results were analyzed with and without the two subjects that developed preeclampsia (PE). Unless specifically stated otherwise the results and p-vales are given for the complete sample size (including PE subjects). If inclusion of the PE subjects impacted significance of results obtained, this was highlighted. Our sample size of 20 subjects was selected to detect a 1.1% difference in delta percent enrichment of ^{57}Fe vs. ^{58}Fe in cord blood with a power of 80% and an alpha level of 0.05 and an estimated attrition rate of 20% using the mean and standard deviation in a prior study of placental non-heme transfer (10). Differences were considered significant if $p < 0.05$.

Results

General characteristics of the study subjects are shown in **Table 4.1**. Of the original 20 subjects, one pregnant adolescent was withdrawn from the study because she did not finish the pork meal; therefore only 19 study participants were followed to delivery. Of these 19 participants, one adult did not show up for the two-week blood draw so Fe absorption data was not available for that subject but maternal and cord blood samples were collected at delivery.

In total 84% (16/19) of the participants had term deliveries (≥ 37 weeks gestation) and 21% (4/19) gave birth to a LBW infant (< 2500 g; mean birth weight of 2408 ± 74 g). Among the 3 preterm births; one adult subject had a spontaneous preterm birth and the other two were induced because they developed PE (one adult/one adolescent). All preterm births resulted in LBW infants. One additional adult with a term pregnancy also gave birth to a LBW infant. There were no differences in primary outcomes between the adult and teen subjects.

Table 4.1

General characteristics of mothers and neonates at delivery[1,2]

Variable	Pregnant (n = 19)
Gestational Age at Delivery (wks)	39.9 ± 1.6 (36.0 - 41.6) [2]
Maternal Age (yrs)	19.0 ± 2.9 (16.0 - 32) [2]
Parity	0 ± 0.9 (0-3) [2]
Pre-pregnancy BMI (kg/m^2)	24.7 ± 7.0 (20.7 - 43.6) [2]
Delivery BMI (kg/m^2)	30.4 ± 7.1 (24.4 – 47.5) [2]
Gestational Weight Gain (lbs)	39.7 ± 15.0 (19-76)[1]
Infant weight (grams)	3169.7 ± 548 (2298-3949) [1]
Race	
African American	53%
Caucasian	47%
Ethnicity	
Hispanic	37%
Non-Hispanic	63%

[1]All values are mean ± SD (range in parentheses)

[2]All values are median ± SD (range in parentheses)

Iron status indicators in mothers and their neonates at delivery are described in **Table 4.2**. No significant differences in the hemoglobin values were evident between the Hemocue vs. the core laboratory value. At delivery 42% of the subjects had depleted iron stores (Hb < 11 g/dL, serum ferritin < 20 μg/L). Approximately 45% of the subjects had non-detectable levels of serum hepcidin at delivery (< 5 ng/mL), whereas all of the neonates had detectable serum hepcidin at birth.

Hepcidin and Iron Status

Maternal serum hepcidin concentrations were significantly associated with multiple indicators of Fe status in the mother at delivery [SF ($p = 0.0001$, $r^2 = 0.59$), TBI ($p = 0.0001$, $r^2 = 0.59$), Hb ($p = 0.01$, $r^2 = 0.31$) and TfR ($p = 0.01$, $r^2 = 0.31$)]. Similarly neonatal serum hepcidin was directly associated with both neonatal serum ferritin (<0.0001, $r^2 = 0.60$) and neonatal total body Fe ($p = 0.0001$, $r^2 = 0.60$). While relationships were evident within the individual neonate or mother at delivery, maternal serum hepcidin was not significantly related to neonatal serum hepcidin or other markers of Fe status in the neonate. Measures of leptin and BMI were not significantly associated with serum hepcidin, placental iron transfer, or iron status in the mother or neonate.

Heme and Non-Heme Iron Transfer to Neonate

There was a significantly greater dose adjusted delta percent enrichment of the ^{58}Fe-heme tracer in cord blood compared to the ^{57}Fe-non-heme tracer enrichment ($p = 0.02$) **Table 4.3**. The differences in Fe transfer between the two isotopes remained significant if presented as percent maternal ingested iron tracer transferred or percent of maternal absorbed tracer transferred to neonate. Similar to our previous data on transfer of non-heme Fe to the fetus (10), no significant relationship was evident between neonatal enrichment of either the heme or the non-heme Fe tracers and the number of days that had elapsed between dosing and delivery.

Table 4.2

Iron status indicators for mother and neonate at delivery

Variable	Maternal (n = 19)	Neonate (n = 19)
Hemoglobin (g/dL)	11.1 ± 1.2 [1, a]	13.4 ± 3.0 [1, b]
< 11 g/dL (% Anemic)	42%	
Serum ferritin (μg/L)	25.4 ± 20.6 [2, a]	134.9 ± 110.2 [2,b]
< 12 μg/L (% Fe Deficiency)	16%	
< 20 μg/L (% Fe Depletion)	42%	
Serum transferrin receptor (mg/L)	5.5 ± 3.3 [2, a]	8.35 ± 2.1 [1, a]
> 8.5 mg/L	21%	
Total Body Iron (mg/kg)	3.4 ± 3.9 [1, a]	8.6 ± 2.7 [1, b]
< 0 mg/kg	16%	
Serum hepcidin (μg/L)	9.3 ± 58.1 [2, a]	61.7 ± 77.0 [2,b]
C-reactive protein (mg/L)	6.1 ± 14.7 [2,a]	0.2 ± 0.15 [2,b]
Folate (nmol/L)	38.0 ± 18.5 [2,a]	69.4 ± 26.6 [1,b]
Vitamin B-12 (pmol/L)	262.2 ± 123 [1,a]	539.5 ± 547 [2,b]
Erythropoietin (IU/L)	24.8 ± 23 [2,a]	29.7 ± 74 [2,a]
Leptin (μg/L)	41.0 ± 22.2 [1,a]	7.3 ± 9.6 [2,b]

[1]All values are mean ± SD

[2]All values are median ± SD

Means that are significantly different from each other are denoted with a different letter ($p < 0.05$)

Table 4.3

Placental Fe Transfer to the Neonate[1]

Variable	Non-heme (^{57}Fe)	Heme (^{58}Fe)	P-value
Δ % enrichment in cord (dose adjusted)[2]	6.9 ± 3.2	8.4 ± 3.2	0.02
% of Maternal Ingested Fe Tracer Transferred to Neonate[3]	2.2 ± 1.4	2.7 ± 1.3	0.02
% of Maternal Absorbed Fe Tracer Transferred to Neonate[4]	4.0 ± 1.6	5.4 ± 2.4	<0.0001

[1]All values are mean ± SD, P-values are provided for paired t-tests

[2] Percent excess was adjusted for the natural abundance of isotope administered and for small differences in dose received.

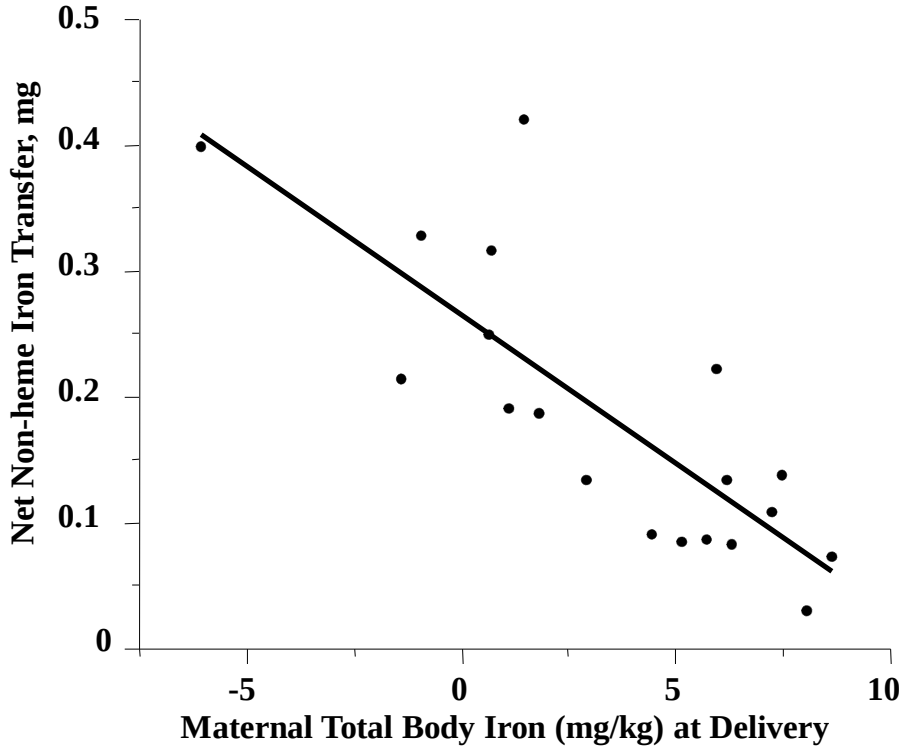
[3] Quantity of tracer dose in neonate divided by average tracer dose administered to mother.

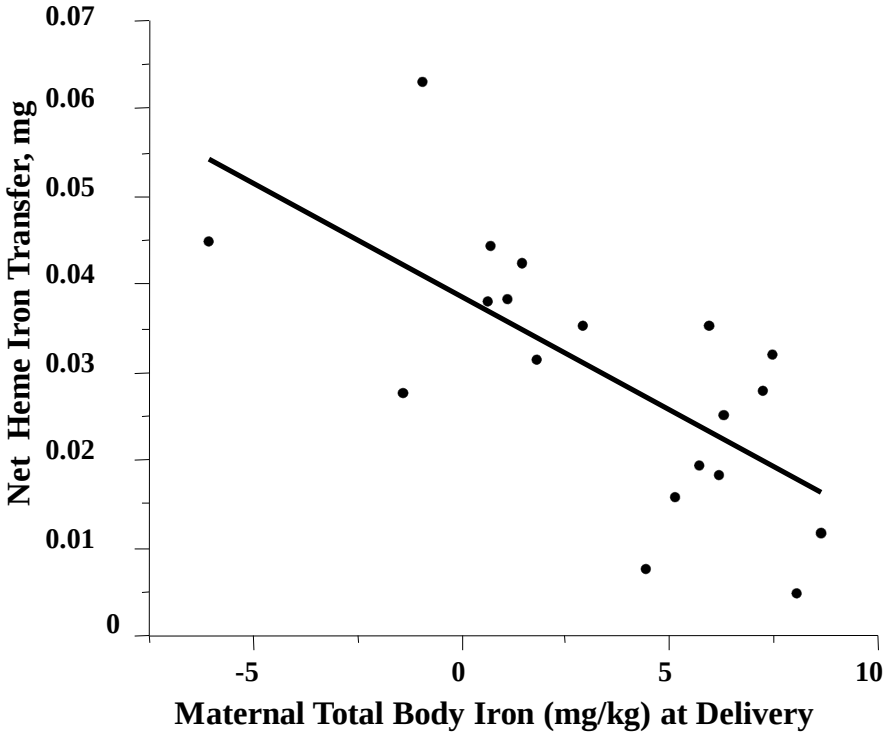
[4] Quantity of tracer dose in neonate as fraction of the absorbed tracer dose.

Relationships between net neonatal non-heme Fe transfer and maternal Fe status were explored. A significant inverse association was found between net non-heme Fe transfer (mg) with maternal serum ferritin ($p = 0.0008$, $r^2 = 0.49$), hemoglobin ($p = 0.005$, $r^2 = 0.38$) and total body Fe ($p<0.0001$, $r^2 = 0.64$) (**Figure 4.2**). Net non-heme Fe transfer (mg) was directly associated with maternal transferrin receptor ($p = 0.0002$, $r^2 = 0.58$) and with maternal percent absorption of non-heme ($p = 0.002$, $r^2 = 0.47$).

Figure 4.2 Net Iron Transfer and Maternal Total Body Iron

Heme (intrinsically labeled ^{58}Fe pork) and non-heme (^{57}Fe as ferrous sulfate) iron were given to 19 pregnant subjects during the third trimester of pregnancy. The net quantity of each tracer in neonatal hemoglobin at birth was assessed from enrichment of cord blood samples. Maternal total body iron (mg/kg) at delivery was inversely associated with both net neonatal non-heme ($p < 0.0001$, $r^2 = 0.64$) and heme ($p = 0.001$, $r^2 = 0.47$) iron transfer (mg).


Net Non-heme Iron Transfer, mg
0.5
0.4
0.3
0.2
0.1
0
-5
0
5
10
Maternal Total Body Iron (mg/kg) at Delivery


Net Heme Iron Transfer, mg
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
-5
0
5
10
Maternal Total Body Iron (mg/kg) at Delivery

Interestingly net neonatal non-heme Fe transfer (mg) was also inversely associated with maternal serum hepcidin at delivery ($p = 0.002$, $r^2 = 0.43$). Pregnant subjects with non-detectable levels of serum hepcidin transferred a significantly greater net quantity of the non-heme tracer (mg) to their neonates than subjects with detectable levels of serum hepcidin ($p = 0.003$). As illustrated in **Figure 4.3** the percent of maternal ingested non-heme Fe tracer transferred to neonate was significantly greater in subjects with non-detectable serum hepcidin ($p = 0.003$). Differences remained significant if percent of maternal absorbed non-heme tracer transferred to neonate was used in place of net mg transferred ($p = 0.03$). Likewise mothers with depleted Fe stores (serum ferritin < 20 μg/L) transferred significantly more non-heme Fe (mg) to the neonate than Fe replete subjects ($p = 0.02$). Net neonatal non-heme Fe transfer was positively associated with maternal EPO ($p = 0.01$, $r^2 = 0.32$). Non-heme Fe transfer was associated with maternal CRP ($p = 0.03$, $r^2 = 0.25$), however if the two subjects with PE (CRP: 66 mg/L, 6 mg/L) were excluded the relationship lost significance ($p = 0.06$). Examination of relationships between net non-heme Fe transfer to the neonate and neonatal Fe status showed that net non-heme Fe transfer was directly associated with neonatal hemoglobin ($p = 0.004$, $r^2 = 0.39$) and weakly associated with neonatal serum ferritin ($p = 0.09$, $r^2 = 0.16$), neonatal total body Fe ($p = 0.1$, $r^2 = 0.13$) and neonatal serum hepcidin ($p = 0.1$, $r^2 = 0.13$). Net non-heme Fe transfer was significantly associated with EPO in the neonate ($p = 0.01$, $r^2 = 0.32$). Using a multiple regression model, 92% of the variance in net of non-heme Fe (mg) transferred could be explained by a model including cord Hb, infant weight and maternal TBI at delivery ($p < 0.0001$, $r^2 = 0.92$). Adding maternal percent non-heme Fe absorption to the model did not explain any additional amount of variation in non-heme Fe transfer and this measure was a non-significant factor in the model after controlling for Fe status.

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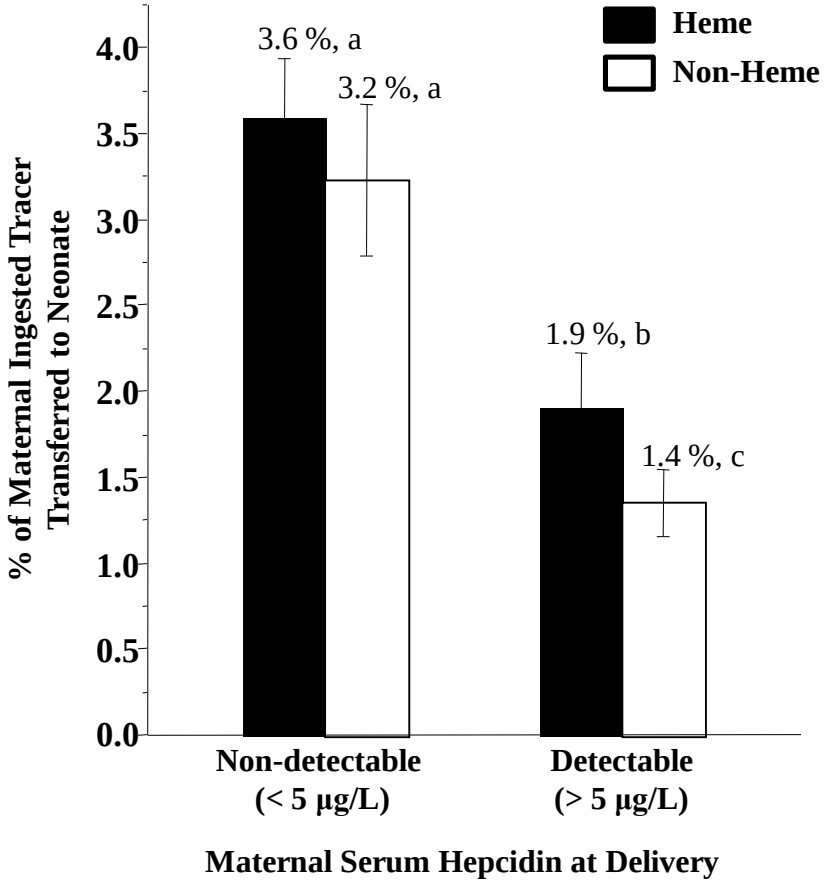

Figure 4.3 Heme and Non-Heme Iron Transfer and Serum Hepcidin

Heme (intrinsically labeled ^{58}Fe pork) and non-heme (^{57}Fe as ferrous sulfate) iron were given to 19 pregnant subjects during the third trimester of pregnancy. The fraction of the total amount of each maternally ingested isotope tracer administered to the mother and transferred across the placenta to the neonate was assessed in 19 neonates. Mothers with non-detectable (< 5 µg/L) serum hepcidin at delivery transferred a significantly greater quantity of both the heme and non-heme tracers to their fetus. Means that are significantly different from each other are denoted with a different letter ($p < 0.05$).

Like net non-heme Fe transfer, the net mg of heme Fe transferred was also significantly associated with both maternal and neonatal Fe status. Net mg of heme Fe transferred was inversely associated with the maternal serum ferritin ($p = 0.003$, $r^2 = 0.41$), Hb ($p = 0.02$, $r^2 = 0.30$) and total body Fe ($p = 0.001$, $r^2 = 0.47$) (**Figure 4.2**) Net neonatal heme Fe transfer (mg) was directly associated with maternal percent absorption of heme Fe ($p = 0.01$, $r^2 = 0.35$) and with TfR ($p = 0.01$, $r^2 = 0.33$) in the mother at delivery. Maternal serum hepcidin at delivery was also inversely associated with net heme Fe transfer (mg) ($p = 0.004$, $r^2 = 0.39$). As with non-heme Fe transfer, mothers with non-detectable serum hepcidin at delivery ($p = 0.002$) and subjects with depleted Fe stores ($p = 0.003$) transferred a significantly greater net quantity of heme Fe to the neonate. Likewise as illustrated in **Figure 4.3** the percent of maternal ingested heme Fe tracer transferred to neonate was significantly greater in subjects with non-detectable serum hepcidin ($p = 0.002$). These differences also remained significant if percent of maternal absorbed heme Fe tracer transferred to neonate was used in place of net mg transferred ($p = 0.008$). Net heme Fe transfer (mg) was weakly associated with EPO in the mother ($p = 0.1$, $r^2 = 0.15$) at delivery. Maternal CRP at delivery was inversely associated with net heme Fe transfer ($p = 0.02$, $r^2 = 0.28$), however when the two PE subjects were excluded the relationship lost significance ($p = 0.052$, $r^2 = 0.23$).

Net neonatal heme Fe transfer was directly associated with neonatal hemoglobin ($p = 0.008$, $r^2 = 0.35$) and was significantly associated with EPO in the neonate ($p = 0.03$, $r^2 = 0.2$) but was not significantly related to other measures of Fe status. Using a multiple regression model, 79% of the variation in net heme Fe transfer (mg) could be explained by cord hemoglobin, infant weight and maternal TBI at delivery ($p<0.0001$, $r^2 = 0.79$). If maternal percent heme Fe absorption was added to the model an additional 10% of the variation could be explained ($p<0.0001$, $r^2 = 0.89$).

Discussion

A significantly greater fraction of maternally ingested or maternally absorbed dietary heme Fe was found in the neonate at birth when compared to that observed for non-heme Fe. These data suggest that there may be differential partitioning of heme Fe once absorbed. Similarities and differences in the determinants of placental transfer of each form of Fe were noted.

Fe status in the maternal-neonatal dyad was inversely associated with the net transfer of non-heme Fe to the neonate at birth. The percent of maternal absorbed non-heme Fe tracer transferred to the neonate (4.1%) was slightly lower than we previously reported for non-heme transfer to the neonate in Fe supplemented (7.0%) and un-supplemented (8.6%) Peruvian women (10). Likewise in an early radioisotope study by Hahn et al., neonates received approximately 7-10% of the Fe absorbed by the mother (17). In our current study, 64% of the variation in non-heme Fe transfer to the neonate measured during the end of the third trimester of pregnancy could be explained solely by maternal total body Fe at delivery. Hepcidin has been demonstrated to impact placental non-heme Fe transport using animal models (13;23). Interestingly, maternal serum hepcidin was associated with placental transfer of non-heme Fe but this hormone only explained 43% of the variation in placental non-heme Fe transfer and the predictive ability of this hormone was no greater than that found using traditional Fe status markers. Moreover, the amount of placental Fe transport explained by hepcidin is similar, but slightly higher than the ~25% reported between hepcidin and intestinal absorption of non-heme Fe (24). Using stepwise regression 92% of the variability in net placental transfer of non-heme Fe was explained by a model that incorporated maternal total body Fe, neonatal hemoglobin, and infant weight. Maternal non-heme Fe absorption was not a significant determinant of neonatal enrichment when maternal iron status was included in the model.

By using both labeled heme and non-heme Fe, similarities and differences in determinants of Fe transfer to the neonate could be explored. Both maternal and neonatal Fe status was significantly associated with the net transfer of heme Fe to the neonate. Similar to non-heme Fe transfer, maternal total body Fe at delivery explained the most variability in neonatal heme Fe transfer but explained less variability for heme Fe transport than for non-heme Fe (47% vs. 64%). As for non-heme Fe, maternal serum hepcidin was also significantly associated with placental transfer of heme Fe but only explained 39% of the variability. A model that incorporated maternal TBI, neonatal Hb, and infant weight explained 79% of the variance in heme Fe transfer. In contrast to relationships observed for non-heme Fe transfer, when heme Fe absorption was added to the model above 89% of the variability was explained. Maternal and/or neonatal Fe status appears to have less of an impact on placental transport of heme Fe than it does for non-heme Fe. This finding mirrors relationships reported in the enterocyte where absorption of heme Fe is also less impacted by Fe stores when compared to non-heme Fe (25).

A greater fraction of the heme Fe dose in the neonate may just indicate greater heme iron intestinal uptake in the mother. However this did not explain the differences noted, as the dose adjusted percent excess, the net mg transferred as a fraction of the maternal ingested tracer and the net mg transferred as a fraction of the amount of Fe absorbed by the mother all supported a preferential placental transfer of heme Fe. Likewise, our study conclusions were comparable if percent enrichment data (directly measured, no assumptions of blood volume or RBC Fe incorporation) were utilized in place of absolute mg or percent of dose values. Absolute values for mg of Fe transferred to the neonate may be impacted by estimations in our calculations. For example, neonatal blood volume was estimated in our study; however since it is a constant factor in calculations it would not influence relative differences observed

between the two forms of Fe. Another limitation in the study design was associated with our ability to assess tracer enrichment only in the RBC; other non-hemoglobin associated neonatal pools are not captured. Because the majority of neonatal Fe (75-80%) (26) is found as circulating hemoglobin the impact of this limitation may not be substantial. Thus, assumptions in our calculations are unlikely to account for the preferential transfer of heme Fe to the neonate during pregnancy.

To date, cellular mechanisms of heme uptake and export are not well characterized. In particular it is not known if all heme is degraded within the cell into inorganic Fe and then exits as inorganic Fe, or if some heme may be exported intact through known heme export proteins (such as Feline leukemia virus subgroup C receptor-related protein, FLVCR and Breast cancer resistance protein, BCRP) in the enterocyte (27). Interestingly both FLVCR (28) and BCRP (29) are most highly expressed in the placenta compared to all other tissues in the body which may suggest that the placenta does utilize heme Fe.

Placental transport of orally administered Fe is impacted by factors that affect cellular transport in both the enterocyte and the placenta. It is interesting to note that while we observed correlations between neonatal heme Fe enrichment and maternal serum hepcidin, we found no significant association between maternal serum hepcidin and maternal utilization of dietary heme Fe (Chapter 3). Previous research has also found that mechanisms of hepcidin regulation may be cell type dependent (30), and detailed mechanistic studies are needed in the placenta to understand the role of hepcidin in regulating heme and non-heme Fe transport across the placenta.

To our knowledge this is the first study to examine relationships between maternal and neonatal serum hepcidin and placental Fe transfer during pregnancy. Serum hepcidin in these pregnant women (9.3 μg/L) was significantly lower than that observed in their neonates at birth (61.7 μg/L). Hepcidin concentrations in our

population are similar but slightly lower than recent maternal (12.4 ng/mL, n = 116) and neonatal (78.4 ng/mL, n = 137) values published by Ervasti et al., in a group of non-anemic women (31). Lower serum hepcidin values may be expected in our pregnant subjects because of the high rates of Fe depletion. In our study population pregnant subjects with non-detectable serum hepcidin (< 5 μg/L) transferred significantly more heme and non-heme Fe to the neonate compared to subjects with detectable serum hepcidin. Similar but non-significant trends were evident between neonatal serum hepcidin and net Fe transfer. Our data support current findings reported in transgenic mice over expressing hepcidin. These mice die shortly after birth due to severe Fe deficiency presumably due to insufficient Fe endowment of birth caused by a deficit in placental Fe flux to the developing fetus (13).

In summary, intrinsically labeled heme may be preferentially transported to the fetus during the third trimester of pregnancy. Maternal hepcidin is significantly associated with placental heme and non-heme Fe transfer. Iron status in the maternal-neonatal dyad significantly impacts transport of both heme and non-heme Fe to the neonate, but a greater percent of the variability of placental heme Fe transfer remains to be fully characterized. Further research on the mechanisms of heme Fe metabolism and mechanisms of placental Fe transfer are warranted.

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CHAPTER 5

IMPACT OF MATERNAL AND NEONATAL IRON STATUS ON PLACENTAL TRANSFERRIN RECEPTOR EXPRESSION[‡]

[‡] **Melissa F Young, Eva Pressman, Marisa Foehr, Thomas McNanley, Elizabeth Cooper, Ronnie Guillet, Mark Orlando, Allison W McIntyre, Julie Lafond, Kimberly O O'Brien. Impact of Maternal and Neonatal Iron Status on Placental Transferrin Receptor Expression. Accepted August 17, 2010; Placenta.**

Abstract

Objective: To elucidate the role of maternal and neonatal iron status on placental transferrin receptor (TfR) expression.

Methods: Ninety-two healthy pregnant adolescents (ages 14-18 years) were followed across pregnancy. Maternal iron status (hemoglobin, hematocrit, serum ferritin, TfR, and total body iron) was assessed in mid-gestation (21-25 wks) and at delivery in the maternal-neonatal dyad. Placental TfR protein expression was assessed via western blot in placental tissue collected at delivery.

Results: Placental TfR expression was inversely associated with maternal iron status at mid-gestation (hemoglobin $p = 0.046$, $r^2 = 0.1$ and hematocrit $p = 0.005$, $r^2 = 0.24$) and at delivery (serum ferritin $p = 0.02$, $r^2 = 0.08$ and total body iron $p = 0.02$, $r^2 = 0.07$). Mothers with depleted body iron stores had significantly greater placental expression of TfR than mothers with body iron stores greater than zero ($p = 0.003$). Neonatal iron status (assessed by serum ferritin in cord blood) was also inversely associated with the expression of placental TfR ($p = 0.04$, $r^2 = 0.06$). Neonates with cord serum ferritin values ≤ 34 μg/L had significantly greater protein expression of placental TfR compared to neonates with cord serum ferritin values > 34 μg/L ($p = 0.01$).

Conclusions: Expression of placental TfR is responsive to both maternal and neonatal iron demands. Increased expression of placental TfR may be an important compensatory mechanism in response to iron deficiency in otherwise healthy pregnant women.

Introduction

Iron deficiency is the most common nutrient deficiency in the world, globally impacting 1.62 billion people with highest rates in pregnant women and children (1). Reduced neonatal iron status at birth has been associated with impaired mental and psychomotor function, altered temperament, impaired neonatal auditory recognition memory and impaired auditory brainstem response (2-5). Therefore, understanding mechanisms of placental iron transport during the third trimester of pregnancy is important to facilitating adequate iron endowment at birth.

The placenta is a key regulatory organ that is essential for fetal nutrient transport. Humans have a hemochorial placenta; maternal blood is in direct contact with the fetal chorionic villi. In the mature hemochorial placenta there are only two layers separating maternal and fetal blood, the syncytiotrophoblast (STB), and fetal endothelial cells (6). The STB is able to selectively regulate transport of oxygen and essential nutrients to the fetus while also allowing for the excretion of fetal waste products (ex. carbon dioxide) to be picked up and cleared by maternal circulation. The majority of fetal iron stores (75 mg Fe/ kg body weight) are acquired during the third trimester of pregnancy (7). Iron must be actively transported across the STB against a concentration gradient in order to meet the fetus's large iron demands. Thus the efficiency of placental iron transfer may set the stage for postnatal iron status and the subsequent risk of developing iron deficiency in infancy. At delivery the neonate typically has accrued a large amount of storage iron with normative serum ferritin concentrations averaging 134 ug/L at a time when most pregnant women have exhausted their ferritin reserves (8).

Previous research from our lab has indicated potential compensatory mechanisms such as increased maternal iron absorption and increased placental iron transport may act to mitigate the risk of iron deficiency in the fetus (9;10, Chapters 3

and 4). The mechanisms and regulatory signals for increased placental iron transport remain unclear. Regulation of key placental iron transporters may allow for greater iron uptake and transfer to the fetus. Some researchers have suggested that this regulation occurs primarily at uptake rather than efflux stages of placental iron transport (11). Transferrin receptor (TfR) is expressed primarily on the apical side of the STB membrane where it is responsible for binding maternal diferric transferrin (12). Previous animal and cell culture studies have demonstrated that maternal iron deficiency leads to increased transferrin receptor mRNA and protein expression (13). Similar upregulation of placental TfR expression has been shown in pregnancies complicated with diabetes (12). However, the role of maternal and neonatal iron status on regulating placental TfR expression is inconclusive in normal healthy populations (14-16). The objective of this research was to assess the determinants of placental TfR expression in response to iron status in a population of healthy pregnant adolescents. Iron demands during pregnancy may be further elevated in this population due to the concurrent iron requirements for growth and development in the adolescent as well as requirements for normal pregnancy and fetal growth. This study provides novel data on placental TfR expression in a vulnerable population at risk for developing iron deficiency.

Subjects and Methods

Subjects

Ninety-two pregnant adolescents (14-18 years) were recruited from the Rochester Adolescent Maternity Program (RAMP) in Rochester New York. Adolescents were eligible to participate if they were otherwise healthy with a singleton pregnancy and planning to deliver at Highland Hospital in Rochester. Adolescents were excluded if they had a diagnosis of HIV infection, diabetes,

preeclampsia, eating disorders, malabsorption diseases, or self-reported drug use. Over 91% of the adolescents reported not smoking during the pregnancy. All adolescents were enrolled in a larger study of maternal and fetal bone health and iron status. Informed written consent was obtained from each subject. The study was approved by the Institutional Review Boards of Cornell University and the University of Rochester and registered with clinicaltrials.gov as NCT01019902 (http://www.clinicaltrials.gov).

A maternal blood sample was obtained between weeks 21-25 of pregnancy (mid-gestation) to examine iron status during pregnancy. Adolescents were followed throughout their pregnancy and at delivery maternal and cord blood samples were obtained to examine iron status in both the mother and neonate. The placenta was collected and processed immediately after delivery. Placental weight (including membranes and cord) and dimensions (length, width, thickness, and volume) were recorded. Representative placental samples were collected, the maternal and fetal membranes removed and sections mixed to provide a representative sampling of placental tissue. The mixed sample was aliquoted, frozen on dry ice and sent to Cornell for subsequent protein extraction. The remaining placenta was then placed in a placental buffer solution (containing DMEM, HEPES, $NaHCO_3$ and 100X Pen/Strep/Neo) and shipped to Cornell for further processing. Representative placental samples were also taken with both the maternal and fetal membranes intact for total iron quantification of whole placenta tissue. Total placental Fe content was analyzed by atomic absorption spectrophotometry (AAS) (PerkinElmer Analyst 800; PerkinElmer Inc, Norwalk, CT). All placental samples were stored at -80°C.

Western Blot Analysis

For protein extraction, placental tissue was kept at 4°C at all times, rinsed in 0.9% saline and homogenized in lysis buffer (containing 1.5 M Tris, 1.0 M $CaCl_2$,

Triton X-100, and protease inhibitor) at ~5000 RPM. Samples were centrifuged for 25 minutes at 14,000g at 4°C and the supernatant was assayed for total protein concentration using the BioRad protein assay method (BioRad, Richmond CA). Supernatants were stored at -80°C until use. Lysates diluted in 6X SDS sample buffer were stored at -20°C prior to Western blot analysis. Protein lysates were run on 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride fluorescence (PVDF-FL) membranes. Protein expression of TfR was analyzed by Western blot analysis using a commercially available antibody (Zymed Laboratores, San Francisco CA). Actin was used as a loading control (Abcam, Cambridge MA). Secondary antibodies (800-goat anti-mouse and 680-goat anti-rabbit) were purchased from Li-Cor Biosciences. Membranes were scanned and analyzed with Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). A placenta control sample was loaded on all of the gels and used to correct for intra-gel variability.

Laboratory Analysis

Hemoglobin (Hb) and hematocrit were analyzed by the Strong Memorial Hospital's clinical laboratory using the Cell Dyn 4000 system. Serum was stored at -80°C prior to analysis. Serum ferritin was measured by an enzyme immunoassay procedure (Ramco Laboratories, Inc Stafford Texas). Serum soluble transferrin receptor was measured with an enzyme linked immunosorbent assay (Ramco Laboratories, Inc Stafford Texas). Total body iron (TBI) was calculated based the formula provided by Cook et al., (total body iron (mg/kg) = -[log (serum transferrin receptor/serum ferritin) – 2.8229]/0.1207) (17).

Data analysis

All statistical analyses were completed using the JMP 8.0 (SAS Institute INC, Cary, NC). Normally distributed data are presented as the mean ± standard deviation and non-normally distributed data are presented as the median ± standard deviation. Variables that were not normally distributed were transformed using the natural log prior to statistical testing. Paired t-tests were used to detect differences within subject groups. Differences between subject groups were compared using a t-test or the Wilcoxon rank sum test for nonparametric data. Linear regression analyses were used to examine the relationships between iron status indicators and placental expression of TfR. Differences were considered significant if $p < 0.05$**.**

Results

Subject characteristics are described in **Table 5.1**. The majority of the adolescent population was African American (64%) and non-Hispanic (71%). For 87% percent of the adolescents this was their first child. Of the pregnant adolescents enrolled in the study only 56% entered pregnancy with a normal BMI (18.5-24.9 kg/m^2); 7% were underweight (18.5 kg/m^2) and 37% entered pregnancy either overweight (> 25 kg/m^2) or obese (> 30 kg/m^2). The majority of adolescents (98%) had term deliveries (≥ 37 weeks gestation) and two adolescents delivered preterm (36.6 and 36.9 wks). One of the preterm infants was also born low birth weight (< 2500 grams) as were two additional term infants. Iron status indicators for the adolescents and neonates are described in **Table 5.2**. Sample sizes varied for each of the biomarkers as limited serum precluded analysis of all indicators in all samples. Furthermore, hemoglobin and hematocrit values were pulled from medical charts retrospectively and were not available for all subjects enrolled in the study.

Table 5.1

General characteristics of the 92 participants[1]

Variable	
Age at enrollment (y)[2]	17.0 ± 1.1 (13.6 – 18.7)
Gestational Age at Delivery (wks) [2]	40.0 ± 1.2 (36.6 – 41.7)
Pre-pregnancy BMI (kg/m^2) [2]	23.2 ± 5.3 (15.2 – 38.7)
Maternal Weight Gain (lbs) [1]	36.4 ± 13.4 (8 – 80)
Infant Weight (g) [1]	3241 ± 499 (2150 – 4705)
Placenta weight (g) [1]	605.3 ± 126.4 (271 – 862)
Race	
African American	64%
Caucasian	35%
Other	1%
Ethnicity	
Hispanic	29%
Non-Hispanic	71%

[1]Mean ± SD is given for normally distributed values (range in parentheses)

[2]Non normally distributed values are given as median ± SD

Table 5.2

Iron status indicators for pregnant adolescents and neonates[1,2]

Variable	Maternal Mid-gestation	Maternal Delivery	Neonate Delivery
Hemoglobin (g/L)	11.3 ± 1.0 [1,a]	11.7 ± 1.5 [1,a]	14.7 ± 1.7 [1,b]
(% Anemic)	19%	29%	
	n = 48	n = 62	n = 32
Serum ferritin (μg/L)	16.5 ± 16.5 [2,a]	17.6 ± 13.0 [2,a]	114.1 ± 81.0 [2,b]
< 12 μg/L	35%	31%	
< 20 μg/L	65%	57%	
	n = 81	n = 88	n = 82
Serum TfR (mg/L)	4.6 ± 2.0 [2,a]	4.6 ± 3.4 [2,b]	8.0 ± 2.4 [2,c]
> 8.5 mg/L	5%	13%	
	n = 81	n = 88	n = 83
Total Body Iron (mg/kg)	3.3 ± 3.7 [2,a]	2.8 ± 3.8 [1,a]	8.7 ± 2.8 [2,b]
< 0 mg/kg	22%	20%	
	n = 81	n = 87	n = 82

[1]Mean ± SD is given for normally distributed values, variables that are significantly different among groups are denoted with a different letter ($p < 0.05$)

[2] Non normally distributed values are presented as median ± SD

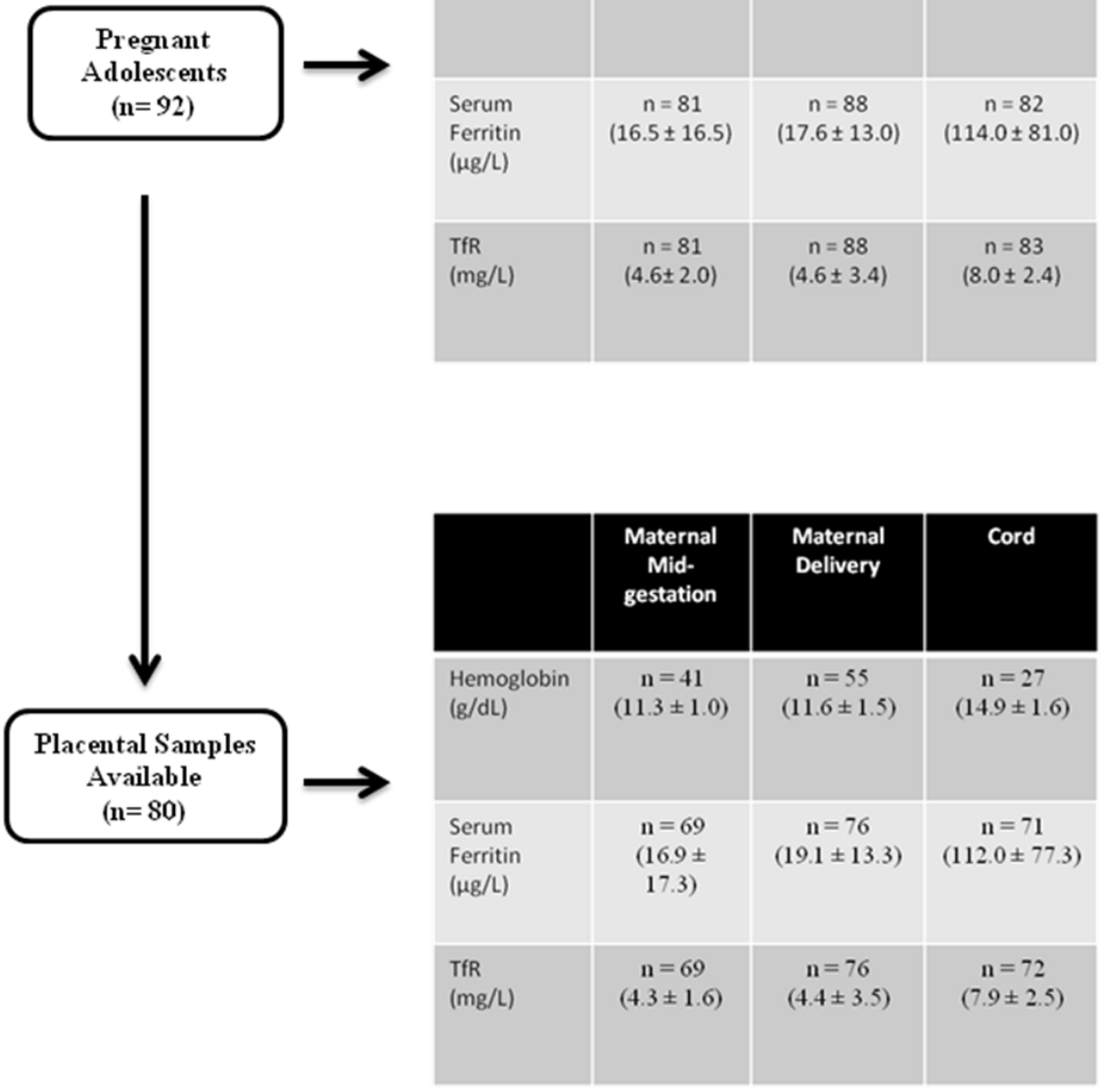
Iron deficiency was prevalent. In the second trimester of pregnancy 19% of the girls were anemic (Hb < 10.5 g/L) and 29% were anemic at delivery (Hb < 11 g/L). In the second trimester of pregnancy 35% were iron deficient (serum ferritin < 12 μg/L) and 65% had depleted iron stores (serum ferritin < 20 μg/L) with similar levels at delivery (31% and 57% respectively). Approximately 20% of the pregnant adolescents had depleted total body iron stores (TBI < 0) during pregnancy and at delivery.

Of the ninety-two adolescents enrolled in the study, eighty placentas (87%) were available for protein analysis. Placentas that were not available were either sent

directly to the pathology department for clinical indications or were not obtained due to difficulties in the logistics of processing shortly after delivery. **Figure 5.1** outlines a flow chart of the enrolled subjects and presents the sample sizes obtained for each of the iron biomarkers in the adolescent and the neonate. Placental TfR protein expression was evident in all placental tissue screened but the expression of the protein was highly variable with a 5-fold difference observed among this cohort. Placental expression of TfR was significantly associated with indicators of maternal and neonatal iron status during pregnancy. Placental TfR expression at delivery was inversely associated with maternal hemoglobin ($p = 0.046$, $r^2 = 0.10$) and hematocrit at mid-gestation ($p = 0.005$, $r^2 = 0.24$). Placental TfR protein expression was also inversely associated with maternal serum ferritin ($p = 0.02$, $r^2 = 0.08$ **Figure 5.2**), and maternal total body iron ($p = 0.02$, $r^2 = 0.07$) at delivery.

Adolescents with iron deficiency at delivery (serum ferritin ≤12 μg/L) had significantly greater placental expression of TfR than those with ferritin levels > 12 μg/L ($p = 0.04$). Likewise teens with depleted total body iron stores (TBI < 0) had significantly greater placental expression of TfR than those with total body iron stores greater than zero ($p = 0.003$, **Figure 5.3**).

At delivery placental TfR expression was also inversely associated with neonatal serum ferritin ($p = 0.04$, $r^2 = 0.06$). Infants with cord serum ferritin concentrations ≤ 34 μg/L ($n = 9$) had significantly greater expression of placental TfR compared to neonates with cord serum ferritin values > 34 μg/L ($n = 75$, $p = 0.01$), **Figure 5.4**. Neonatal serum ferritin was also directly associated with maternal iron serum ferritin at mid gestation ($p = 0.0006$, $r^2 = 0.16$) and at delivery ($p = 0.04$, $r^2 = 0.05$). A model that included both maternal and neonatal serum ferritin at delivery explained 12% of the variability in protein expression ($p = 0.01$, $r^2 = 0.12$).



	Maternal Mid-gestation	Maternal Delivery	Cord
Hemoglobin (g/dL)	n = 48 (11.3 ± 1.0)	n = 62 (11.7 ± 1.5)	n = 32 (14.7 ± 1.7)
Serum Ferritin (µg/L)	n = 81 (16.5 ± 16.5)	n = 88 (17.6 ± 13.0)	n = 82 (114.0 ± 81.0)
TfR (mg/L)	n = 81 (4.6± 2.0)	n = 88 (4.6 ± 3.4)	n = 83 (8.0 ± 2.4)

	Maternal Mid-gestation	Maternal Delivery	Cord
Hemoglobin (g/dL)	n = 41 (11.3 ± 1.0)	n = 55 (11.6 ± 1.5)	n = 27 (14.9 ± 1.6)
Serum Ferritin (µg/L)	n = 69 (16.9 ± 17.3)	n = 76 (19.1 ± 13.3)	n = 71 (112.0 ± 77.3)
TfR (mg/L)	n = 69 (4.3 ± 1.6)	n = 76 (4.4 ± 3.5)	n = 72 (7.9 ± 2.5)

Figure 5.1 Flow Chart of Available Samples.

Iron status indicators were assessed in a cohort of ninety-two pregnant adolescents. Of these 92 adolescents, 80/92 (87%) had placental tissue collected at delivery. The corresponding charts illustrate the iron status biomarkers available for data analysis.

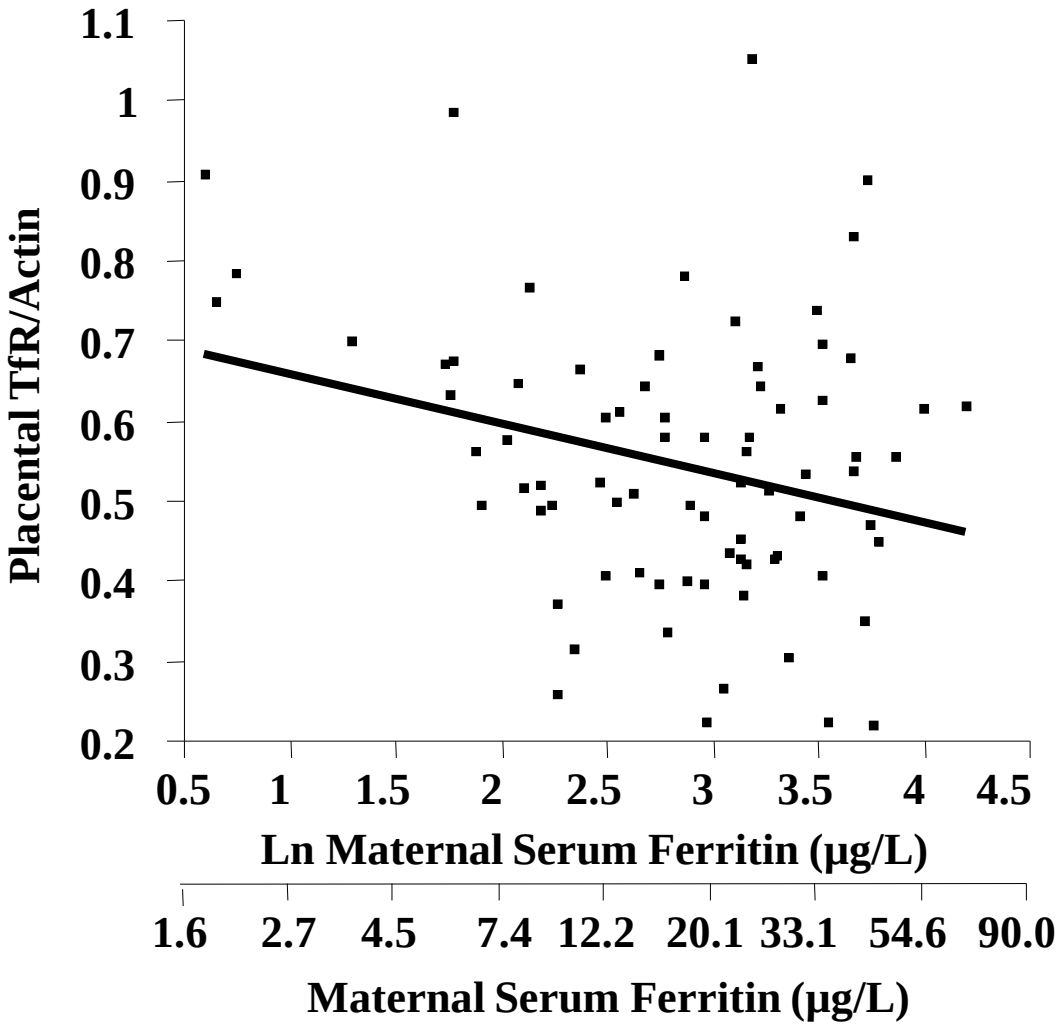


Figure 5.2 Placental TfR Expression and Maternal Serum Ferritin.

Placental expression of transferrin receptor (TfR) was examined in relation to maternal serum ferritin concentrations at delivery in a group of 76 pregnant adolescents (ages 14-18 years). Placental TfR expression at delivery was inversely associated with maternal serum ferritin concentrations ($p = 0.02$, $r^2 = 0.08$).

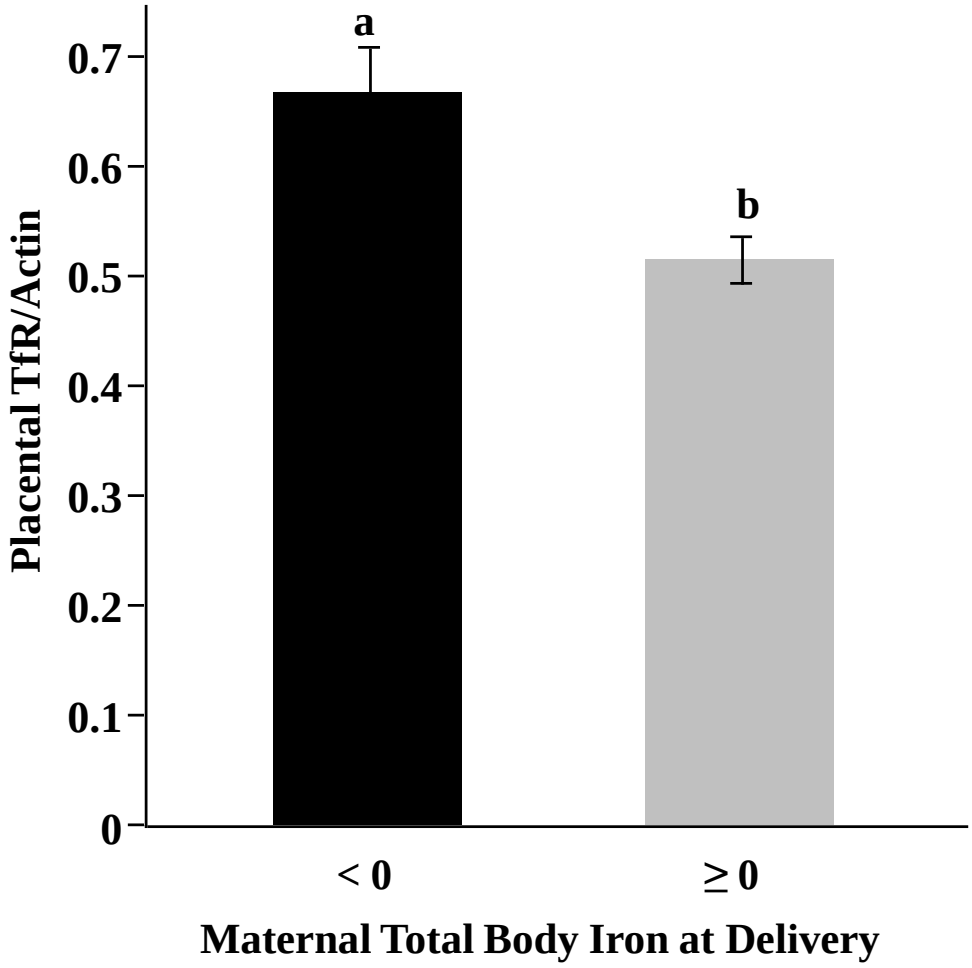


Figure 5.3 Placental TfR Expression and Maternal Total Body Iron.

Placental expression of transferrin receptor (TfR) was examined in relation to maternal total body iron at delivery in a group of 75 pregnant adolescents (ages 14-18 years). Adolescents with depleted total body iron stores at delivery (n = 14) had significantly greater placental expression of TfR when compared to adolescents with total body iron stores greater than zero (n = 61, p = 0.003).

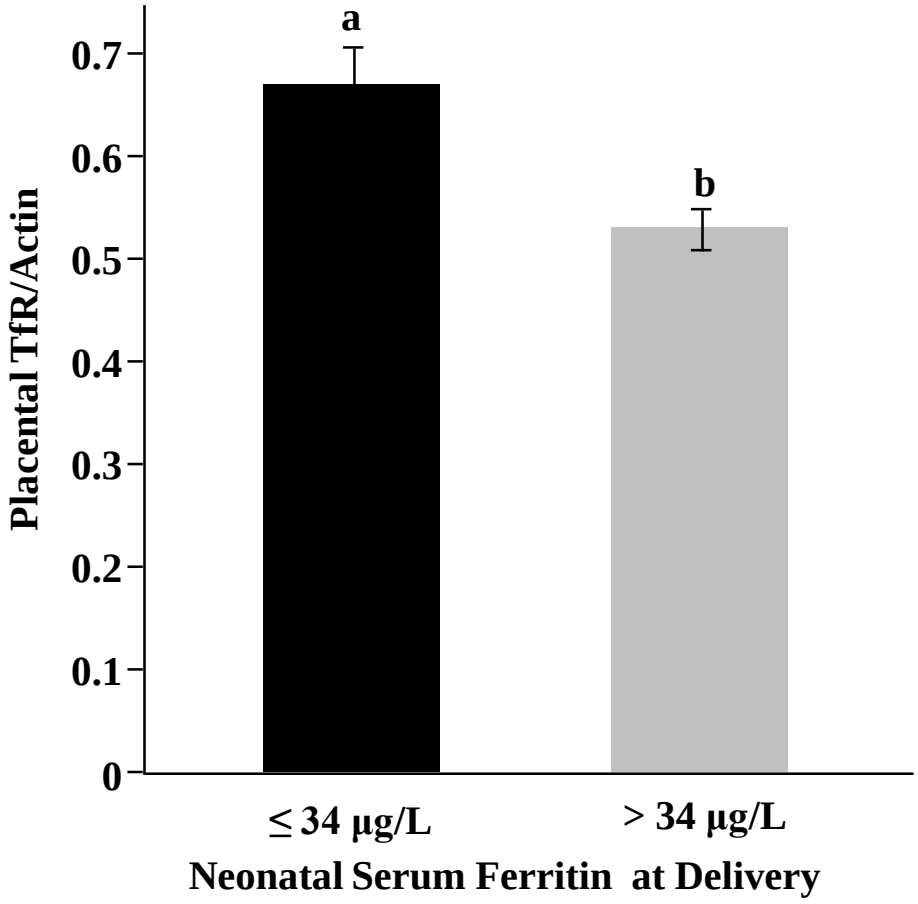


Figure 5.4 Placental TfR Expression and Neonatal Serum Ferritin.

Placental expression of transferrin receptor (TfR) was examined in relation to neonatal serum ferritin concentrations at delivery (n = 71). Infants with cord serum ferritin concentrations ≤ 34 µg/L (n = 6) had significantly greater protein expression of placental TfR (n = 65, p = 0.01).

Interestingly, the expression of placental TfR was also inversely associated with placental volume ($p = 0.003$, $r^2 = 0.22$) and placental thickness ($p = 0.02$, $r^2 = 0.14$) but was not significantly associated with placental weight or placental iron concentration. Placental TfR was not significantly different in adolescents that self-reported smoking during pregnancy. The average use of cigarettes among those that reported smoking during pregnancy was approximately 8.6 cigarettes per day (ranging from 1 – 20 cigarettes per day). Neonatal hemoglobin was slightly but significantly elevated in neonates whose mothers smoked during pregnancy (14.5 ± 1.7 vs. 15.9 ± 0.8, $p = 0.03$). However, maternal iron status, infant weight, placental weight, gestational age and other markers of iron status in the neonate were not significantly impacted by maternal smoking status.

Discussion

Placental TfR protein expression was responsive to both maternal and neonatal iron demands. Our research confirms data in animal and cell culture models as well as that reported from complicated pregnancies in which neonatal iron status was known to be adversely impacted (12;13). Our data are unique in that they confirm these relationships among a relatively healthy population of adolescents with uncomplicated pregnancies. Previous data obtained in healthy adult populations have been inconclusive and were primarily undertaken among much smaller study cohorts (14-16). Research by Bradley et al., in twenty-one healthy non-anemic women found placental TfR expression to be unrelated to neonatal or placental iron status (as measured with cord serum ferritin and placental ferritin) (14). Likewise, in a group of thirty-eight healthy women that included both iron replete and iron deficient (~20%) pregnant women, placental TfR expression was unrelated to maternal iron status (hemoglobin, TfR, and serum ferritin); in that study neonatal iron status was not

reported (15). In a group of forty healthy pregnant women, relationships between protein expression of TfR and maternal iron status approached significance, but a significant increase in placental TfR mRNA was evident in subjects with mild anemia (90 g/L ≤ Hb < 110 g/L) and no change in those with moderate anemia (Hb < 90 g/L) compared to non-anemic women (16). Our study population was a large cohort of pregnant adolescents. The racial composition of this group is reflective of the increased risk of early childbearing found among US minority populations. In total 65% of the group was African American and 29% was Hispanic. Adolescents are at increased risk of anemia because of the additional demands of maternal growth superimposed on those required for the developing fetus. These differences in study design and iron status of our population may have allowed us to detect significant differences in placental TfR expression.

Maternal and neonatal iron stores were significantly associated with placental TfR and explained approximately 8% and 6% of the variability in protein expression of placental TfR, respectively. A unique aspect of this study is that iron status was assessed in the maternal-neonatal dyad whereas many previous studies focused only on the neonate or the mother. While it is difficult to tease apart the independent role of maternal versus neonatal iron status, when both maternal and neonatal iron status are included in a model, substantially more of the variability in protein expression (12%) was explained compared to use of either maternal or neonatal status alone. In women with depleted total body iron at delivery (TBI < 0) a 29% increase in placental expression of TfR was noted compared to mothers with total body iron stores greater than zero. Likewise, there was a 26% increase in the expression of placental TfR in the placentas of neonates born with cord serum ferritin values ≤ 34 μg/L. In spite of these significant associations, the measures of iron status we utilized only explained a relatively small fraction of the almost five-fold difference noted in relative protein

expression. In research by Li et al., there was an approximate three-fold increase in placental TfR mRNA in subjects with mild anemia compared to non-anemic pregnant women but only a ~20% non significant increase in placental TfR protein expression (16). Because the majority of variability in placental TfR was not explained by iron status, other factors that have been linked to placental TfR expression such as IRP-1 (18) and hepcidin (19) warrant further investigation.

Despite evidence of upregulation of placental TfR in response to reduced iron stores, this compensatory mechanism may not be sufficient to achieve optimal iron stores in the neonate at birth. Although no clear cut offs exist to define suboptimal neonatal iron stores, our cord serum ferritin values can be compared to values that have been associated with impaired functional outcomes in other populations. Approximately 9% of the neonates in this study were born with cord serum ferritin values ≤ 34 μg/L, a level reported to be suspected of brain iron deficiency (2). Siddappa et al., reported that infants born to diabetic mothers with cord serum ferritin ≤ 34 μg/L had impaired infant auditory recognition memory as well as lower psychomotor development scores after one year compared to infants born to diabetic mothers with cord serum ferritin > 34 μg/L (2). This is in accordance with previous research in infants of mothers with diabetes who were born with reduced liver, heart and brain iron (12;20) despite increased placental TfR expression (12). Thus the placenta's ability to upregulate placental iron transport to compensate for limited iron availability may not be sufficient to endow the neonate with optimal iron stores in the face of suboptimal maternal iron status.

In summary, in an otherwise healthy group in whom nutrient needs are likely challenged by increased maternal needs coupled with pregnancy demands we found placental TfR protein expression to be responsive to both maternal and neonatal iron status. Regulation of placental TfR expression may be an important compensatory

mechanism to increase iron flux to the developing fetus. More research is needed to understand the role and regulation of other placental iron transporters in response to maternal and neonatal iron status.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

Summary

The research presented provides novel insight into some of the key determinants of heme and non-heme iron absorption and placental iron transfer in a group at high risk for iron deficiency. There were four key specific aims of this research that were described in Chapters 2-5, respectively. Below is a brief summary of the specific aims, outcomes and implications of the findings generated.

For many years the systemic hormone responsible for whole body iron homeostasis had not been identified. This changed in 2000 when the small peptide hepcidin was identified (1). Because this was only a 25 amino acid peptide, the small size made it difficult for assays to accurately measure this hormone. For these technical reasons, many researchers measured prohepcidin, a 60-AA precursor of hepcidin, as marker for hepcidin expression. My first specific aim was to assess relationships between serum hepcidin and serum prohepcidin with non-heme iron absorption from an iron supplement and an orange flesh sweet potato source in a group of healthy non-pregnant women. Using stable iron isotopes and a newly developed assay for hepcidin we published the first data demonstrating a significant inverse relationship between hepcidin and non-heme iron absorption (2). Of interest, only ~25% of the variability in non-heme iron absorption was explained by this hormone, a similar amount of variability can be explained simply with the use of iron stores (serum ferritin). This finding was published in the *American Journal of Clinical Nutrition* along with an editorial highlighting its significance (3), and was subsequently replicated by other research groups (4;5). Serum prohepcidin, on the other hand, was not significantly related to measures of iron absorption or serum hepcidin and may not serve as a useful substitute for serum hepcidin.

My second specific aim was to build on the results of the first study and examine the impact of iron status and serum hepcidin on both heme and non-heme

iron absorption in non-pregnant and pregnant women. Stable isotopes were again utilized to assess iron absorption from heme and non-heme iron sources (^{58}Fe-intrinsically labeled heme and ^{57}Fe as ferrous sulfate). This was the first human study to my knowledge to assess heme iron metabolism during pregnancy and little is known about the role of serum hepcidin in regulating heme iron absorption. Pregnant and non-pregnant women absorbed significantly more iron from the heme-based iron meal compared to absorption from the non-heme source. Serum hepcidin was inversely associated with non-heme iron absorption but was unrelated to absorption of heme iron. Absorption from the non-heme iron source was also more sensitive to iron stores than heme iron. Heme iron from animal based foods provided a highly bioavailable source of dietary iron during pregnancy. The lack of association of iron stores and serum hepcidin with heme iron absorption, yet strong correlations with non-heme iron absorption, highlights the differential mechanisms of transport of heme versus non-heme iron across the enterocyte.

My third specific aim was to elucidate determinants of heme and non-heme placental iron transfer to the fetus over the last trimester of pregnancy. Pregnant women that had received the iron isotopes during the third trimester of pregnancy were followed to delivery. At parturition, data on iron status and serum hepcidin in the maternal-neonatal dyad were examined in relation to isotopic enrichment in the cord blood. Significantly more heme iron was transferred to the neonate than non-heme iron, even after accounting for the amount of iron absorbed by the mother. Net neonatal non-heme and heme iron transfer (mg) were significantly related to iron status in the maternal-neonatal dyad and inversely associated with maternal serum hepcidin. This study provides novel findings that there may be preferential fetal utilization of maternally ingested heme iron compared to non-heme iron during pregnancy. This implies differential pathways of heme versus non-heme iron entry

into the placenta. For this to occur it suggests that at least some dietary heme iron not only enters but also exits the enterocyte intact through separate mechanism as proposed in Figure 1.1.b in Chapter 1. This may also explain why a significant relationship was observed between serum hepcidin and absorption of non-heme iron but not heme iron. Hepcidin's primary mechanism of impacting iron absorption is binding to ferroportin (the sole indentified non-heme iron exporter in the enterocyte) leading to its internalization and degradation. If some of the heme iron were to exit the enterocyte intact through the heme exporters that are known to be present in the enterocyte it may explain the lack of association with serum hepcidin and the preferential transfer of heme iron to the neonate. However it is possible that there is a combination of Figures 1.1.a and 1.1.b and further research is needed on the cell type specific mechanisms of hepcidin in the enterocyte and the placenta.

My final specific aim was to assess determinants of placental TfR protein expression in the placenta. Limited data previously existed on the placenta's ability to upregulate iron transporter expression in response to iron status in a healthy population. In a large group of ninety-two healthy pregnant adolescents, expression of placental TfR was inversely associated with iron status in the maternal-neonatal dyad. Increased expression of placental TfR may be an important compensatory mechanism in response to iron deficiency during pregnancy.

Based on the combined results of Chapters 2-5, conceptual frameworks (**Figure 6.1.a and Figure 6.1.b**) for non-heme iron and heme iron metabolism during pregnancy have been developed to summarize results.

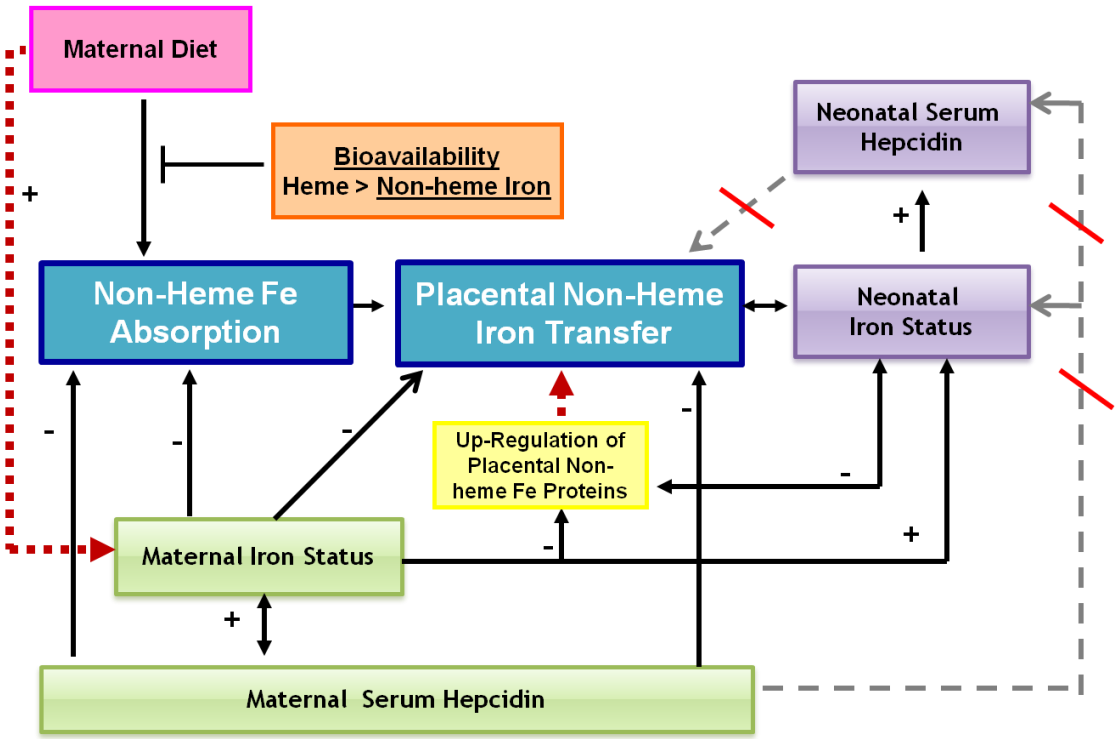

Figure 6.1.a Non-heme Iron Metabolism Conceptual Framework

Solid black lines represent significant relationships that have been presented in previous chapters. Gray dashed lines with red slashes represent non-significant relationships that have been presented in previous chapters. The red dotted lines represent presumed or hypothesized relationships that were not directly tested with this research.

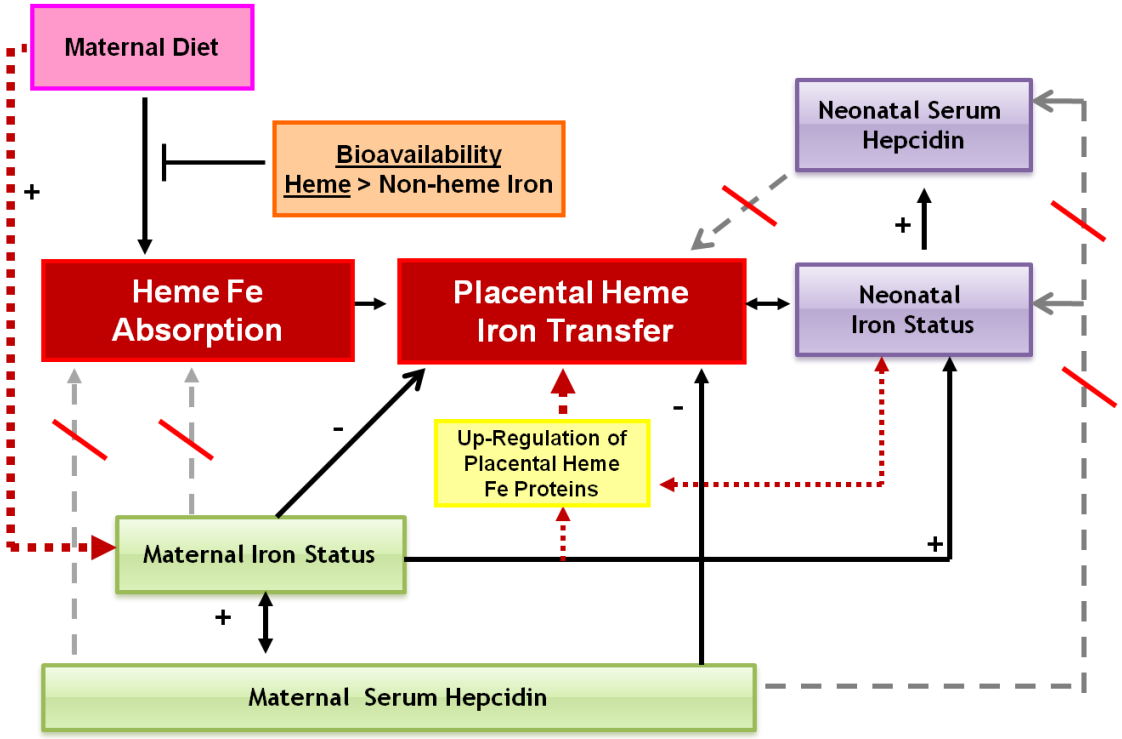

Figure 6.1.b Heme Iron Metabolism Conceptual Framework

Solid black lines represent significant relationships that have been presented in previous chapters. Gray dashed lines with red dashes represent non-significant relationships that have been presented in previous chapters. The red dotted lines represent presumed or hypothesized relationships that were not directly tested with this research.

In brief, from Figure 6.1.a non-heme iron absorption was dependent on the bioavailability of the meal (greater iron absorption from the liquid iron supplement compared to the orange flesh sweet potato meal). Non-heme iron absorption was inversely associated with maternal iron status and serum hepcidin, which are in turn were correlated, but iron status appeared to be the major factor influencing non-heme iron absorption. It is hypothesized that the diet in turn will directly impact the iron status of the individual. Non-heme iron transfer was also inversely correlated with both maternal iron status and serum hepcidin. Again maternal iron status was the primary determinant of non-heme iron transfer, explaining the majority of the variability. Non-heme iron transfer was also associated with neonatal iron status which was in turn correlated with the mothers iron status. Although neonatal iron status was highly correlated with neonatal serum hepcidin, neonatal serum hepcidin was not significantly correlated with iron transfer or maternal serum hepcidin. Altered iron stores in both the mother and neonate were able to increase the expression of placental TfR. Although, not directly tested, it is hypothesized that the increased expression of key placental iron transport proteins in turn acts to increase net flux to the fetus. Likewise we see a similar picture for heme iron metabolism (Figure 6.1.b) with two key exceptions. Heme iron absorption was not significantly associated with maternal iron status or serum hepcidin although these factors do appear to directly impact placental heme iron transport. The expression of heme iron transporters in the placenta were not assessed, however a similar trend is hypothesized. Although Figures 6.1.a and 6.1.b are simplified and other factors including age, CRP and EPO have been shown be correlated with some of the variables, the diagrams still give a good basic outline of the primary relationships driving iron metabolism examined in this research. This data provides novel information on heme iron metabolism, serum hepcidin and mechanisms of upregulating iron transfer during pregnancy.

Future Directions

Based on the results of this study further research is warranted to investigate molecular mechanisms of heme iron absorption and placental iron transport in order to elucidate the pathways of preferential absorption and transfer of heme iron. Only one iron transport protein in the placenta was examined in this research and future studies should examine the role of other non-heme and heme iron transporters (ferroportin, DMT1, FLVCR, hemopexin receptor, etc) not only with respect to protein expression but also RNA expression. Furthermore, molecular studies on how hepcidin regulates iron metabolism during pregnancy are also needed.

Despite increased iron absorption, placental expression of iron transport proteins and iron transfer to the neonate, many mothers and infants still had suboptimal iron status at birth. Over 50% of the mothers had depleted iron stores at delivery and in the large cohort of pregnant adolescents (Chapter 5) almost 10% of the neonates were born with cord serum ferritin values ≤ 34 μg/L. Further research is needed to design interventions to prevent the onset of iron deficiency during pregnancy in order to optimize iron stores at birth. Compliance with prenatal iron supplementation is often a problem in this teen population. Qualitative studies on the barriers for adherence would provide valuable insight in how to improve consumption of supplements during pregnancy. The addition of heme iron sources to the diet may provide a highly bioavailable iron source, but studies on availability, access and acceptance are required. Furthermore, research on the functional consequences of iron deficiency during pregnancy on both the mother and neonate is necessary; as pregnant teens may be at elevated risk due to the high iron demands of pregnancy concurrent with the iron demands of the growing adolescent.

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