

UTILIZATION OF HEME AND NON-HEME IRON SOURCES DURING PREGNANCY

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Intestinal iron absorption increases during pregnancy to support maternal and fetal iron demands. Although knowledge on non-heme iron absorption has advanced substantially since identification of the hormone hepcidin in 2000, the mechanisms of heme iron absorption remain elusive. Catabolism of senescent red blood cells releases 10-times more iron into the circulation daily than does iron absorption from the diet, yet the contribution of this endogenous maternal heme iron source to fetal iron transfer is unknown. The human placenta abundantly expresses an array of heme transporters but it is unclear whether these proteins play a role in placental heme utilization. The objective of this research was to explore the mechanism and regulation of heme and non-heme iron utilization in the duodenum and the placenta, the two major sites of iron flux during pregnancy.

To address these questions, stable iron isotopes (^{57}Fe and ^{58}Fe) were used to measure duodenal heme and non-heme iron absorption in rats and placental transfer of iron derived from maternal red blood cell catabolism and from maternal diet in pregnant women. In Sprague Dawley rats, hepcidin up-regulation suppressed the absorption of both heme and non-heme iron but the effect was more pronounced for non-heme. Hepcidin was inversely associated with iron transporters on the apical but not basolateral side of the duodenum, suggesting apical iron transport is the primary target of hepcidin action. The stable iron

isotope study in pregnant women (n=16, ages 17-35 years) indicated that iron derived from maternal red blood cells was transferred to the fetus, revealing the importance of maternal red cell iron stores in supporting fetal iron demands. In a cohort of pregnant adolescents (13-18 years), placental protein expression of two putative heme transporters were associated with neonatal iron status, consistent with a role of these proteins in placental iron transport. Future research is needed to elucidate the roles of these transporters in the uptake and intracellular trafficking of heme in the placenta and to characterize the sources of heme iron in the circulation and inter-tissue heme trafficking pathways.

BIOGRAPHICAL SKETCH

Chang Cao was born in a small city in Sichuan province in southwest China in 1988, and lived there until moving to the provincial capital Chengdu for high school. After graduating from high school, Chang was accepted to the University of Hong Kong and spent her freshman year as an exchange student at Zhejiang University in Hangzhou, China before relocating to Hong Kong in 2006. In her junior year, Chang studied at Tufts University in Boston as an exchange student and became interested in organic agriculture. She decided to focus her undergraduate thesis research on prospects of developing organic agriculture as a means of poverty reduction in western China and obtained a research fellowship to conduct field and laboratory experiments at the China Agricultural University in Beijing in the summer of 2009 where she compared the nutritional and microbiological qualities of organically and conventionally grown green peppers.

In December 2009, Chang graduated from the University of Hong Kong with first class honors and came to Cornell to pursue a PhD degree in Nutrition. At Cornell, Chang has supported several courses in the Division of Nutritional Sciences as a teaching assistant and conducted animal and human research on the mechanisms of heme iron utilization under the mentorship of Dr. Kimberly O'Brien.

After her graduate studies, Chang will pursue her interest in heme iron research in the laboratory of Dr. Mark Fleming's at Boston Children's Hospital. Chang enjoys movies, detective novels, art, travel, and meeting people. She loves food and has a big appetite.

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LIST OF ABBREVIATIONS

| | |
|-------|--|
| BMI | Body mass index |
| CRP | C reactive protein |
| DMT1 | Divalent metal transporter 1 |
| Dcytb | Duodenal cytochrome b |
| EPO | Erythropoietin |
| Fe | Iron |
| FLVCR | Feline leukemia virus subgroup C receptor |
| FPN | Ferroportin |
| Hb | Hemoglobin |
| Hct | Hematocrit |
| HFE | Human hemochromatosis protein |
| Hp | Haptoglobin |
| Hx | Hemopexin |
| ID | Iron deficiency |
| IDA | Iron deficiency anemia |
| IL6 | Interleukin 6 |
| IRE | Iron responsive element |
| LRP1 | Low density lipoprotein receptor-related protein 1 |
| PCFT | Proton coupled folate transporter |
| RBC | Red blood cell |
| SF | Serum ferritin |
| sTfR | Soluble transferrin receptor |
| TBI | Total body iron |
| TfR | Transferrin receptor |

CHAPTER 1

INTRODUCTION

Literature review: Pregnancy and iron homeostasis¹

Introduction

Iron deficiency (ID) and iron deficiency anemia (IDA) remain significant public health concerns both in the US and throughout the world (1). It is estimated that 2 billion women and children worldwide are ID (2). Pregnant women are particularly vulnerable to IDA due to the high iron (Fe) demands of pregnancy. Fe requirements increase markedly over pregnancy from a net savings early in pregnancy (due to the cessation of menstruation), to a maximum 3-8 mg of Fe per day during the third trimester of pregnancy (3). The net Fe cost of a singleton pregnancy has been estimated to range from 480-1,150 mg, of this nearly 300 mg is deposited in the fetus (3). Pregnancy often results in a depletion of existing maternal Fe stores, every 1 µg of circulating ferritin represents 10 mg of storage Fe (4). To accommodate the Fe demands of pregnancy, women would need to enter pregnancy with approximately 500 mg of storage Fe, yet only 20% of reproductive aged women are estimated to have this reserve and approximately 40% of women worldwide enter pregnancy with no storage Fe (5).

The large numbers of reproductive-aged women with pre-existing anemia coupled with the elevated Fe requirements of pregnancy make pregnant women particularly vulnerable to IDA. In the US, 30% of pregnant women have a total body iron (TBI) < 0 mg/kg in the third trimester of pregnancy, indicative of exhausted Fe stores (6). National data suggest that the prevalence of anemia is 21.55 per 1,000 women (7). Similar data from a largely minority

¹ Cao C, O'Brien KO. Pregnancy and iron homeostasis: an update. *Nutr Rev* 2013;71(1):35-51.

population reports a prevalence of IDA of 27.4% in the third trimester of pregnancy (8). The prevalence of anemia is impacted by use of prenatal supplements and ranges from 14-52% in pregnant women that are not taking prenatal supplements, to 0-25% among pregnant women consuming Fe supplements (9).

The magnitude and consequences of IDA during pregnancy are well appreciated yet many unresolved questions remain on the physiology of Fe absorption from all dietary sources of Fe (heme and non-heme), the impact of supplement type (prenatal vs. single or multiple micronutrient) on maternal and neonatal Fe status, the optimal timing and net amount of supplemental Fe needed across pregnancy and the full impact of Fe supplementation on birth outcomes. Appropriate Fe status indicators are needed in order to evaluate the impact of Fe supplementation on maternal Fe status yet assessment of Fe status during pregnancy is compromised by variability in maternal plasma volume expansion (PVE). Relationships between maternal Hb and adverse birth outcomes are often U-shaped with increased risk observed at both ends of the Hb spectrum (10, 11). The degree to which increased Hb may be a consequence of failure to expand plasma volume requires further study as increased Fe stores are not always evident in those with elevated Hb concentrations (12). At this time, there are no rapid or validated approaches that can be utilized to control for variability in PVE across pregnancy.

Little attention has been focused on the impact of maternal Fe status during pregnancy on neonatal Fe status at birth due to challenges and concerns with routine blood sampling among healthy newborns. Recent studies are beginning to compile reference data on hematological status and Fe stores among healthy newborns using both venous and cord blood (13-15). The goal of this document is to review issues relevant to Fe utilization during pregnancy and its impact on the Fe endowment of the neonate at birth considering Fe availability from both

maternal diet (heme and non-heme sources) and from endogenous maternal Fe reserves (Fe stores and red blood cell (RBC) catabolism).

Fe supplementation across pregnancy

Fe supplementation is routinely recommended for US pregnant women to help meet the increased Fe demands of pregnancy. In 2008, the American Congress of Obstetricians and Gynecologists (ACOG) recommended prenatal supplementation with an Fe-containing prenatal supplement for all US pregnant women (16). ACOG also advocates universal anemia screening with additional targeted Fe supplementation for pregnant women that are found to be anemic (16). Similarly, the EU guidelines, also recommend daily Fe supplementation during the second half of pregnancy (17). However, in the most recent National Institute of Clinical Excellence guidelines on antenatal care in the UK (18), universal Fe supplementation is not advocated for all pregnant women on the basis of inconsistent reports on the benefits of Fe supplementation on maternal and infant health as well as potential drawbacks including gastrointestinal side-effects, reduced absorption of non-heme Fe (19), and other minerals (20), as well as increased oxidative stress (8). According to the UK guideline, Fe supplementation should only be considered for women with Hb concentrations < 11 g/dL in the first trimester or Hb concentrations < 10.5 g/dL at 28 weeks of gestation (18). The belief that Fe supplementation is not warranted in all women is also evident by the lack of any pregnancy related increase in the reference dietary intakes for Fe in the UK (21) or EU (17).

In contrast, the recent *2010 Dietary Guidelines for Americans* emphasized the need for women of reproductive age to consume Fe-rich food sources and components that enhance Fe absorption in addition to foods containing heme Fe because it is more readily absorbed (22). These dietary guidelines also recommended that all pregnant women consume a daily Fe

supplement as recommended by their health care provider (22). While the 2010 Dietary Guidelines for Americans highlighted the intake of heme Fe sources because of their enhanced bioavailability, few data on mechanisms of heme absorption and utilization are available, especially among pregnant women.

Many studies have reviewed the impact of maternal non-heme Fe supplementation on risk of anemia across pregnancy and found Fe supplementation to result in positive benefits for both maternal and neonatal outcomes (23-27). An updated Cochrane Review on effects and safety of daily Fe supplementation during pregnancy has been published and supports that an increase in maternal Hb concentrations and a decrease in the risk of anemia is evident in women receiving either intermittent or daily Fe/folate supplementation (24). In general, daily oral Fe (30-60 mg of elemental Fe) and folic acid (0.4 mg) supplementation have been found to reduce the risk of low birth weight infants, increase birth weight, and in some groups impact neonatal mortality (28-31). The positive impact on birth outcomes has been found to occur both with (28, 30, 32), or without (29, 31) a significant impact on maternal Hb in the third trimester.

In many settings, multiple micronutrient deficiencies exist and treatment with Fe alone may adversely impact absorption and status of other micronutrients that are limited in the diet. In particular, numerous studies in pregnant and non-pregnant females have found interactions between Fe and zinc absorption (33-36). In 1999, UNICEF and WHO suggested that 15 additional micronutrients (at recommended nutrient intake levels) be added to a 30 mg Fe supplement in part to enhance the absorption and utilization of Fe (37). Recent reviews of randomized controlled trials evaluating the impact of multiple micronutrient supplements compared to Fe-folate supplements alone found that multiple micronutrient supplements containing Fe had no significant additional benefit on prevention of maternal anemia during the

third trimester when compared to Fe-folate supplements alone (27, 38, 39). There was, however, evidence of a reduction in risk of low birth weight (38), and small for gestational age births (27, 39), compared to Fe-folate supplements alone.

Maternal response to non-heme Fe supplementation across pregnancy is influenced by baseline Hb concentrations as highlighted in a recent Fe supplementation study (40). In this study, over 1200 pregnant women from Burkina Faso were randomized to receive either a standard Fe-folate supplement (60 mg of Fe and 400 µg of folic acid, n = 632) or a UNIMMAP multiple micronutrient supplement containing 30 mg of Fe and 400 µg folic acid in addition to 13 other micronutrients (n = 636) (40). The study uniquely examined treatment response as a function of Hb status at entry into the study (anemic vs. non-anemic). Among non-anemic women, Hb concentrations decreased across gestation irrespective of supplementation group. In contrast, Hb concentrations increased significantly across gestation in those that were anemic at baseline, such that by term the mean Hb was not significantly different between those that were anemic vs. non-anemic at baseline, nor did type of supplement ingested impact treatment response (40). Of interest, the risk of hemoconcentration (Hb > 13 g/dL) was associated with micronutrient intake in the cohort as a whole, but was independent of supplementation group or initial maternal Hb concentration. This study highlighted the adaptable physiology of Fe absorption during pregnancy and the ability of maternal Fe status to impact utilization of Fe from supplemental, non-heme Fe sources during pregnancy. The study also highlighted the physiological decrease in Hb that occurs over pregnancy. Even with Fe supplementation, 51% of women remained anemic at term, highlighting the regulation of Fe utilization in response to maternal needs. More data is needed to understand the risk of hemoconcentration and the physiological determinants of this process in women taking Fe supplements.

The majority of Fe supplementation studies have provided inorganic, non-heme Fe supplements which are known to be tightly regulated in response to maternal Fe stores and circulating hepcidin concentrations (33, 41). Far less is known about the relative bioavailability or efficacy of heme Fe during pregnancy. To date, only one published randomized controlled study has evaluated the impact of a supplemental heme Fe source on Fe stores in a group of 90 pregnant women (42). Women were randomized to receive ferric fumarate alone (27 mg with ascorbic acid), ferric fumarate and heme Fe (24 mg of ferric fumarate with 3 mg of heme Fe as Hemofer®) or a placebo. Supplements were ingested from week 20 of pregnancy through 24 weeks postpartum. Fe supplementation over the second half of pregnancy was associated with a significant improvement in Hb compared to the placebo group. However, the heme group had significantly better Fe stores at the end of pregnancy compared to the placebo and heme intake appeared to protect against depletion of maternal Fe stores at 24 weeks postpartum compared to the placebo or ferric fumarate group alone (42).

Few studies on maternal Fe intake have differentiated non-heme from heme Fe and little is known about the effect of heme Fe intake on maternal and fetal health. In a recent survey in 1,274 British pregnant women, intake of non-heme, but not heme Fe, was positively associated with infant birth weight (43). Median heme Fe intake in these women during the first trimester was 0.3 mg/day, which was lower than that reported among a recent US pregnant cohort (0.8 mg/day) (43). In this US cohort, the risk of gestational diabetes mellitus increased in those with higher heme intake in early pregnancy even after controlling for consumption of red and processed meats, saturated fat, and cholesterol (43). More large-scale studies among pregnant women are needed to examine the impact of dietary heme Fe intake on other aspects of maternal and neonatal health as well as on Fe status.

Genome-wide association studies have recently been utilized to identify the proportion of variation in common Fe status markers that is explained by genetic determinants. Using this approach, genetic variants in the HFE and transferrin genes explain roughly 40% of the genetic variation in serum transferrin (44, 45). Significant, but much smaller associations have been found between single nucleotide polymorphisms in TPRSS6 (Matripase-2) and serum Fe, transferrin saturation and erythrocyte mean cell volume (44). Matripase-2 is involved in the regulation of Fe homeostasis via its ability to suppress the stimulation of hepcidin transcription as mediated by bone morphogenic protein (46). A recent stable Fe isotope absorption study in mother-toddler pairs found that only approximately 50% of non-heme Fe absorption was explained by Fe status and known dietary factors and a highly significant relationship was observed between individual mother-toddler pairs after controlling for known confounders (47). Based on these observations, the authors speculated that much of non-heme Fe absorption was driven by inheritance or by an unknown factor in the shared environment of the mother and her child (47). As this field evolves, it may be possible to identify and target those at increased risk for anemia so that appropriate interventions or increased anemia surveillance can be implemented among pregnant women at increased risk for anemia.

Assessing maternal Fe status during pregnancy

A number of common biomarkers are utilized to characterize anemia and/or maternal Fe status across pregnancy including; Hb (Hb) and hematocrit (Hct), serum ferritin (SF), serum soluble transferrin receptor (sTfR), and total body iron (TBI). A list of common indicators and their reference ranges are presented in **Table 1.1**. At present, Hb and Hct are the most widely used indicators to assess anemia during pregnancy. Measures of Hb and Hct drop from early to

mid-pregnancy as a result of pregnancy-associated hemodilution, i.e. a smaller increase in RBC mass (~25%) relative to that of plasma volume (~50%) (48).

Table 1.1. Cut-off values for ID, and normal ranges of Fe status indicators in pregnant women ¹

| Indicator | First trimester | Second trimester | Third trimester |
|--|-----------------|------------------|-----------------|
| Hb cut-off (g/dL) | | | |
| Cut-off used to define anemia | < 11.0 | < 10.5 | < 11.0 |
| Cut-off used for anemia in black women | < 10.2 | < 9.7 | < 10.2 |
| Cut-off used for anemia in cigarette smokers | < 11.3 | < 10.8 | < 11.3 |
| Cut-off used for anemia in those living at high altitude | < 11.2–13.0 | < 10.7–12.5 | < 11.2–13.0 |
| Reference range | 11.6–13.9 | 9.7–14.8 | 9.5–15.0 |
| Hct cut-off (%) | | | |
| Cut-off used to define anemia | < 33.0 | < 32.0 | < 33.0 |
| Cut-off used for anemia in black women | < 31.0 | < 30.0 | < 31.0 |
| Cut-off used for anemia in cigarette smokers | < 34.0 | < 33.0 | < 34.0 |
| Cut-off used for anemia in those living at high altitude | < 33.5–39.0 | < 32.5–38.0 | < 33.5–39.0 |
| Reference range | 31.0–41.0 | 30.0–39.0 | 28.0–40.0 |
| SF (µg/L) | | | |
| Reference range | 6.0–130.0 | 2.0–230.0 | 0–116.0 |
| Serum sTfR (mg/L) | | | |
| sTfR/SF | > 4.4 or > 8.5 | | |
| sTfR-F index (sTfR/log SF) | > 300.0 | | |
| TBI (mg/kg) | > 1.8 | | |
| Reference range | < 0 | | |
| Serum Fe (µg/dL) | 5.6–7.3 | 3.4–5.0 | 0.8–2.0 |
| Transferrin saturation (%) | < 40.0 | | |
| TIBC (µg/dL) | < 16.0 | | |
| ZnPP (µmol/mol heme) | > 400.0 | | |
| | > 70.0 | | |

¹ Cut-offs and ranges were taken from the following references: Mei et al. (6), ACOG (16), Suominen et al. (49), Abbassi-Ghanavati et al. (50), CDC (51), Carriaga et al. (52), and van den Broek et al.(53). Reference ranges are values between the 2.5 and 97.5 percentiles of the reference populations.

Because of the considerable interpersonal variation in the degree of hemodilution and PVE (54), pregnant women with the same RBC mass may have very different Hb and Hct values. Factors that have been shown to impact PVE in humans or animals include: preeclampsia (55), chronic hypertension (56), idiopathic fetal growth restriction (57), ID (58), food restriction

(59), and low protein intake (60). The impact of these conditions on the Fe status indicators should be kept in mind when interpreting biochemical data across pregnancy.

The have published Hb and Hct thresholds indicative of gestational anemia based on the 5th percentile of the distributions of these two variables and cutoff values for anemia for pregnant women during the first, second and third trimester (61). Several population-based studies have shown optimal pregnancy and birth outcomes in women with Hb concentrations between 9.5-11.0 g/dL (62-65), raising the possibility that the current CDC norms for Hb may be set too high (66). In addition, high Hb concentrations (> 13 g/dL) have also been shown to confer an increased risk of low birth weight and pre-term delivery and this U-shaped relationship between Hb and adverse birth outcomes has been observed in pregnant populations from many diverse demographic backgrounds (10, 11, 62-64). This association has been suggested to be in part due to shared pathologies including hypertensive disorders and preeclampsia (67).

While Hb/Hct measures are diagnostic of anemia, a large proportion of anemia is due to causes other than ID such as folate or vitamin B₁₂ deficiencies, hematological disorders, and chronic inflammation (68). More specific Fe status indicators are necessary to determine whether the anemia noted is a consequence of ID. SF is one such indicator that is commonly used to estimate storage Fe because of its close correlation with both total mobilizable tissue Fe reserves (69), and Fe stores in bone marrow (70). The WHO has identified SF as the best indicator of the effectiveness of Fe intervention trials, and proposed the use of a SF value $< 12-15$ $\mu\text{g/L}$ to indicate absent Fe stores in adults (61). Most studies in pregnant women have adopted the lower end of this range, i.e. 12 $\mu\text{g/L}$, to account for the physiologic decline in SF due to PVE (6, 71). This cut-off (SF < 12 $\mu\text{g/L}$) is also one of the discriminatory criteria set by the Institute of Medicine for prescribing Fe supplements during the first and second trimester of pregnancy (72).

However, similar to other Fe status indicators in blood, SF is subject to the impact of hemodilution and is further limited by its poor sensitivity to true Fe depletion due to its large within-person variation (73), and the Fe-independent rise this acute phase protein exhibits as a consequence of inflammation. This inflammatory response has been shown to mask ID in individuals with heightened inflammatory status (74, 75). Pregnancy itself is an inflammatory state with many studies detailing elevations in inflammatory markers such as C-reactive protein (CRP) (76, 77), GM-CSF (78, 79), leptin (80), and T-helper 2 cell cytokines (interleukin-4, interleukin-6 and interleukin-10) (81, 82). Early pregnancy and parturition are thought to be pro-inflammatory states, while mid-gestation is thought to be an anti-inflammatory state (83, 84). Thus, a concurrent measurement of SF with other acute phase reactants such as CRP and alpha 1-acid glycoprotein is generally recommended to control for possible effect of inflammation (61). Body mass among non-pregnant women has been linked with suboptimal Fe stores and elevated CRP even after adjusting for Hb concentrations (85). This is thought to be a consequence of down-regulation of Fe absorption from elevated hepcidin concentrations that occur due to adiposity associated inflammation (85). The ability of pregnancy itself, and/or pregnancy associated weight gains to impact systemic inflammatory markers, hepcidin and Fe status is poorly studied.

While SF is an indicator of body Fe stores, sTfR yields information about cellular Fe demands and the erythropoietic proliferation rate. Circulating sTfR is derived primarily from the detachment of the receptor from developing erythroblasts and serum concentration of this indicator rises when Fe supply to the erythron is limited due to exhausted Fe stores (86). In the absence of erythropoietic disorders, serum sTfR identifies mild tissue Fe insufficiency with high sensitivity (87). In contrast to SF levels, serum sTfR exhibits low intra-personal variability (73),

and is less affected by acute phase phenomenon (86, 88); therefore, serum sTfR is particularly useful in identifying ID in individuals with normal to high SF during inflammation (88). Serum sTfR concentrations remain stable in the first trimester of pregnancy and increase progressively from late pregnancy to term, a phenomenon often attributed to increased erythropoiesis and higher occurrence of tissue ID as gestation progresses (71, 89, 90). Studies that have evaluated the utility of serum sTfR in pregnant women reported a sensitivity of 70%-78% and specificity of 46%-100% of high sTfR concentration in detecting ID as diagnosed by other Fe indices (71, 89, 91). In adults, a serum sTfR > 8.5 mg/L is indicative of tissue ID as determined by quantitative phlebotomy (87). This cut-off has been shown to be highly specific to Fe depletion during pregnancy (52), and has been used by most published literature on sTfR during pregnancy (89, 92). However, a recent publication using National Health and Nutrition Examination Survey data (2000-2006) used 4.4 mg/L as the upper reference level for sTfR in pregnant women based on the manufacturer's reference range in 261 women without ID or anemia (6). The overall prevalence of ID defined by sTfR > 4.4 mg/L (17.4%) was significantly lower than that identified when using SF < 12 µg/L (25%) and this difference remained evident when examined within each trimester (6). However, when ID was evaluated in reference to absent TBI (< 0 mg/kg), this sTfR cutoff was shown to be able to identify a similar number of ID cases with reasonable sensitivity (64.0%) and high specificity (92.8%). There is a large amount of variability observed between different commercial TfR immunoassays (93), but a new WHO reference standard, prepared from recombinant TfR, is now available for data harmonization purposes (94).

Assessment of both SF and sTfR may better capture the full spectrum of tissue Fe homeostasis and when expressed as a ratio (sTfR/SF), may avoid correct for PVE differences

and thus provide a more accurate reflection of Fe status during pregnancy. The sTfR/SF ratio as a measure of ID was first proposed by Skikne et al. in 1990 (87). This measure has been tested against bone marrow aspirates in a group of anemic pregnant women and showed 85% sensitivity and 79% specificity when the cutoff was set at 300 (53). Use of both sTfR and SF data also allows for an estimation of TBI using the logarithm developed by Cook et al. which was calibrated by quantitative phlebotomy in healthy adults to provide a continuous, numerical description of Fe status from surplus to deficit (93). It should be noted that because TBI is expressed on a weight basis, the expected increase in body weight during pregnancy may underestimate the true body Fe stores in pregnant women when comparing the values to non-pregnant populations. Although the validity of TBI cannot be tested by phlebotomy in pregnant populations, this model of Fe status assessment has been utilized in several pregnancy studies (6, 12, 93), including the recent National Health and Nutrition Examination Survey (1999-2006) (6). The cut-off of TBI for tissue ID is set at 0 mg/kg in pregnant women as in other adult populations (6, 93).

Hepcidin, the key Fe-regulatory hormone secreted by the liver, negatively affects systemic Fe homeostasis by blocking both non-heme Fe absorption from the diet and Fe mobilization from macrophages and hepatocytes (95). Hepcidin production is induced by Fe stores and inflammation and is suppressed by erythropoietic activity and hypoxia (96). Due to the lack of standardized quantification methods and the scarcity of large population-based studies to derive reference ranges (97), hepcidin assays performed to date are restricted to research situations and this measure has unknown diagnostic utility among pregnant women. One recent pregnancy-specific, reference study for serum hepcidin was published in a group of 116 Fe-replete pregnant women at term (98). The reported 95% CI ranged from undetectable (< 5

ng/mL) to 58.6 ng/mL, with a median of 10.7 ng/mL. This study also included 75 pregnant women with low Fe stores (SF < 12 µg/L) but did not attempt to determine cut-offs of hepcidin for use in ID diagnosis. The utility of urinary hepcidin has also been examined in 190 pregnant Bangladeshi women and was found to be strongly associated with SF (99). We recently explored the role of hepcidin in regulating absorption of heme and non-heme Fe in a small sample of pregnant and non-pregnant women (41). Heme Fe absorption was significantly higher than that observed for non-heme Fe in both pregnant and non-pregnant women (41). Of interest, serum hepcidin only became a significant predictor of non-heme Fe absorption when data from the pregnant and non-pregnant participants were combined, explaining 24% of the variation in non-heme Fe absorption, a value similar to that we previously reported for non-pregnant women (100). This raises the question of whether hepcidin provides additional information about Fe status when compared to the existing panel of Fe status indicators in individuals absent of pathologies, and if yes, whether this additional information would offset the high analytic cost of the test. It is worth noting that although the stimulatory effect of inflammation on hepcidin is well characterized (101), different inflammatory markers seem to exhibit differential relationships with hepcidin during pregnancy (102), which may be reflect the differences in the temporal response to the induction of inflammation of these acute phase proteins. This illustrates the need for future research to identify biomarkers that are most useful in the interpretation of Fe status measures known to be affected by inflammation. Also, due to methodological differences in the analytical approaches currently available for hepcidin measurement, widely different absolute values are obtained which limits the comparability of data. A recent review of analytical differences indicates that while absolute differences exist, relative differences are comparable between the existing hepcidin quantification methods (103). Furthermore, there is conflicting

evidence as to whether urinary hepcidin is a better estimate of hepcidin production than serum hepcidin (104-106). Serum hepcidin measures have lower variability (96), and would not be impacted by pregnancy associated alterations in glomerular filtration rate, urine production and do not need to be corrected for creatinine. More pregnancy data are needed to assess the diagnostic utility of hepcidin in relation to the existing panel of Fe status indices across gestation.

Placental Fe transport

All the Fe required for fetal growth and development is actively transported from the mother to the fetus by the placenta. Early radiotracer studies in animals with hemochorial placentas (like the human which has a hemochorial placenta), suggest that Fe is rapidly moved from the maternal circulation to the fetus against a concentration gradient (107-109). Studies in the rabbit (107), and rhesus monkey (110), have injected ⁵⁹Fe radiotracer directly into the fetus *in vivo*. Lack of measurable radioactivity in maternal plasma (110), or non-dosed littermates (107), suggest that the transfer of Fe to the fetus is unidirectional and that little to no recycling of Fe occurs. Kinetic studies using perfused human placenta lobules also support the *in vivo* animal data and indicate that this transfer is unidirectional (111). However, recently the heme export protein, Feline Leukemia Virus subgroup C Receptor (FLVCR)1, was identified and found to be most abundantly expressed in the human placenta when compared to other body tissues (112). This may suggest a reverse flow of heme Fe from the placenta to the maternal circulation that perhaps functions to protect the feto-placental unit from Fe toxicity. In erythroid cells export of heme Fe by FLVCR1 is an important mechanism utilized to prevent cellular heme toxicity and ensure cell survival (112). The presence of FLVCR1 in the placenta, and recent data linking this protein to maternal Fe status (113), suggest that this protein may serve a similar role in the

placenta. More experimental evidence is needed to assess the role of FLVCR1 in relation to placental Fe transport.

While data on the mechanisms of transport are evolving over time, it is known that a net flux of roughly 270 mg of Fe is transferred across the placenta to be accumulated by the developing fetus, most of which occurs over the last 10 weeks of gestation (114). During late pregnancy, an average of 5.6 mg of Fe per day from dietary or endogenous maternal sources is trafficked across the placenta to meet fetal demands (3). The magnitude of this placental Fe flux during late gestation is nearly 6-times higher than the amount of Fe typically absorbed across the enterocyte from dietary sources in non-pregnant women, and represents nearly 30% of the 20 mg of Fe that is typically catabolized daily from senescent RBCs. The Fe delivered to the fetus is obtained either from three primary sources as detailed in **Figure 1.1**: 1) dietary Fe sources (heme and non-heme Fe); 2) liver Fe stores; or 3) Hb in RBCs.

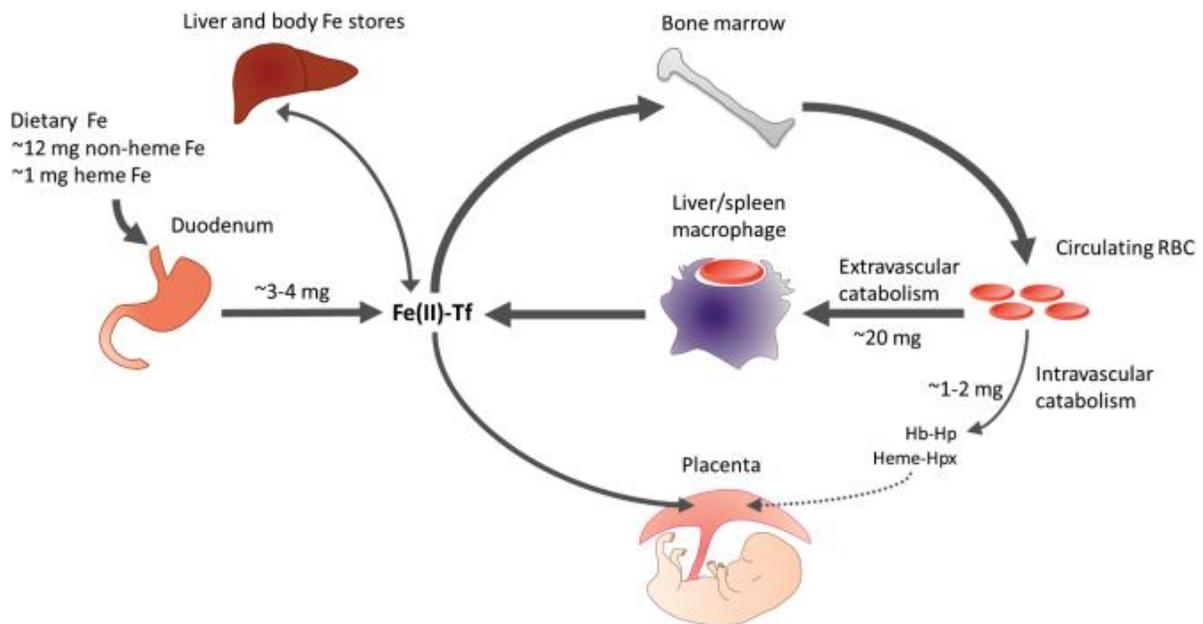


Figure 1.1. Fetal Fe sources during pregnancy

Placental non-heme Fe transport

The majority of research on cellular Fe trafficking has focused on the enterocyte. It is assumed that the placental syncytiotrophoblast employs a similar Fe trafficking system to that of the duodenal epithelium but trafficking mechanisms in placental tissue are less well characterized. Several lines of evidence, including the identification of placenta-specific ferroxidase necessary for basolateral Fe efflux (115), and the demonstration of the redundancy of divalent metal transporter 1 (DMT1) in mouse placenta (116), cast doubt on this claim and support the notion that the placenta may have a distinct cellular Fe process that uniquely responds to the local and systemic maternal and fetal regulatory signals. Furthermore, the placenta, unlike the enterocyte, cannot rid excess intracellular Fe by sloughing of senescent crypt cells into the lumen of the gastrointestinal tract every 3 days. To avoid excess Fe transfer to the fetus the placenta must restrict uptake into the syncytiotrophoblast, store unwanted Fe within the tissue until parturition, or export excess intracellular Fe back into maternal circulation if not needed by the developing fetus. Fe stable isotopic data suggests that once transferred to the fetus there is no recycling of non-heme Fe from fetal to maternal circulation based on data that find no association between days post-dosing and enrichment of the neonate at birth (117-120). Unlike the enterocyte that responds only to systemic maternal signals, the placenta may regulate expression of proteins in response to both maternal and fetal signals. The fetus begins producing hepcidin in the first trimester of gestation (121), and neonatal hepcidin concentrations are regulated independently of maternal hepcidin concentrations (98). Further work assessing relative associations between placental transport proteins and both maternal and neonatal Fe status at birth is needed to elucidate this process.

While many steps in the trans-placental Fe trafficking await further characterization, initial uptake of Fe from the circulation into placental tissue is relatively well defined. Classic thought holds that the majority if not all fetal Fe is derived as inorganic Fe from maternal diferric-transferrin (122-124). The serum diferric-transferrin binds to placental transferrin receptor (TfR) which is abundantly expressed on the apical side of placental syncytiotrophoblast, leading to the endocytosis of the vesicle containing the transferrin-TfR complexes. Acidification of the vesicle facilitates the dissociation of Fe from transferrin. The released Fe is reduced to its ferrous state by a recently identified family of ferrireductases, Steap 1, 2, 3, 4 (125), and transported out into the cytoplasm through a protein channel by DMT1, though there is evidence of an DMT1-independent Fe exit pathway functioning in the placenta (116). Both the Steap 3 and 4 ferrireductases are highly expressed in the placenta and may exhibit functional redundancy to maintain Fe flow to the fetus (125). Once in the cytoplasm, Fe is either incorporated into its storage form (ferritin), or is delivered to transferrin in the fetal circulation via concerted action of the Fe exporter, ferroportin (FPN) and the placental ferroxidases such as Zyklopen (115).

Research from both human and animal models has revealed several steps in the placental Fe transfer pathway that may be subject to regulation by maternal and fetal Fe status. Previous research from our laboratory has shown that the amount of dietary non-heme Fe transported across the placenta is increased in women with ID, suggesting that Fe transport apparatus in the placenta is able to adapt to maternal Fe supply (117, 118). In rats fed a low Fe diet, compensatory changes in the placental Fe transport pathway were seen with increased activity of copper oxidase as well as enhanced expression of TfR and the iron-responsive element (IRE)-regulated DMT1 but not FPN (126). These authors further demonstrated a commensurate rise in radio-Fe uptake with increased TfR mRNA expression in cultured BeWo cells, suggesting that

the up-regulation of proteins involved in placental Fe transfer is partly responsible for the functional increase in Fe transfer during deficiency (126). The few available human data also confirm this feedback control of placental Fe transfer by demonstrating an inverse relationship between maternal Fe stores and placental expression of TfR (127) and ferritin (128).

The importance of fetal signals in controlling placental Fe transport is an evolving area of research. In a series of experiments in rats, Gambling et al. (121) identified the fetal liver Fe as the key fetal determinant of placental Fe transfer. A strong inverse association between fetal liver Fe and placental expression of TfR was evident suggesting that fetal hepcidin may be a key mediator of this regulation (121). Interestingly, the authors found no relationship between hepcidin and the expression of its ligand FPN in the placenta and suggested that the classic interaction between hepcidin and FPN observed in other tissues may not be operative in the rat placenta (121). Our data in pregnant teenagers also found a significant inverse association between neonatal SF and placental TfR protein expression even after controlling for maternal SF (127).

Placental heme Fe transport

Several diverse types of placentas, across many species (carnivores in particular) have paraplacental structures on the margins of the placenta which are referred to as hemophagous organs (129). Within the hemophagous organ maternal erythrocytes are ingested by phagocytic trophoblast cells and the heme Fe released is utilized to meet fetal Fe demands (129). In some species, such as the dog, these areas stain bright green due to the magnitude of Fe accumulated at this site (130). Humans have a hemochorial placenta; most species with this type of placenta are believed to rely primarily on uptake of transferrin-bound Fe to meet fetal Fe requirements (129). However, the human placenta also exhibits an abundant expression of many proteins involved in

heme Fe uptake and utilization including proton coupled folate transporter (PCFT) (131, 132), FLVCR1, and FLVCR2. The FLVCR1 protein functions as a heme export protein (113, 133), while FLVCR2 may serve as a heme influx protein (134). In humans, expression of FLVCR1 is the highest in the placenta when compared to all other body tissues (112, 135), perhaps as noted above this is suggestive of a putative use of maternal heme as source of fetal Fe during pregnancy. The human placenta also contains the necessary scavenger receptors for heme- and Hb-complexes (136, 137). Much of daily RBC catabolism occurs extravascularly in the liver and spleen (releasing non-heme Fe for subsequent use by tissues) but 10-20% of RBC catabolism is thought to occur intravascularly which would release 1-2 mg Fe into the circulation as free Hb and heme (138). These substances are highly reactive and are quickly bound to their respective blood carriers haptoglobin (Hp) and hemopexin (Hx) (139), to be ferried to target tissues (hepatocytes and reticuloendothelial macrophages) known to express scavenger receptors for the Hb- and heme-carrier complexes. Interestingly, the scavenger receptors for Hb (the Hp-Hb complex binds to CD163) (137), and heme (Hx-heme complexes bind to lipoprotein-related protein 1 (LRP1)) (136), are also abundantly expressed in the human placenta, supporting the notion that the placenta may be capable of utilizing heme Fe sources. Heme Fe may also be obtained from dietary absorption. The mechanisms by which heme Fe is absorbed and exported across the enterocyte have not yet been determined. It was initially thought the PCFT was the elusive heme transporter but this protein was subsequently identified as a proton coupled folate transporter and its affinity for folate was found to be more than two orders of magnitude greater than its reported affinity for ⁵⁵Fe-hemin (132). The heme export protein (FLVCR1) is also abundantly expressed in the human duodenum (112). A summary of the proteins involved in heme and non-heme Fe utilization by the placenta and enterocyte are depicted in **Figure 1.2**.

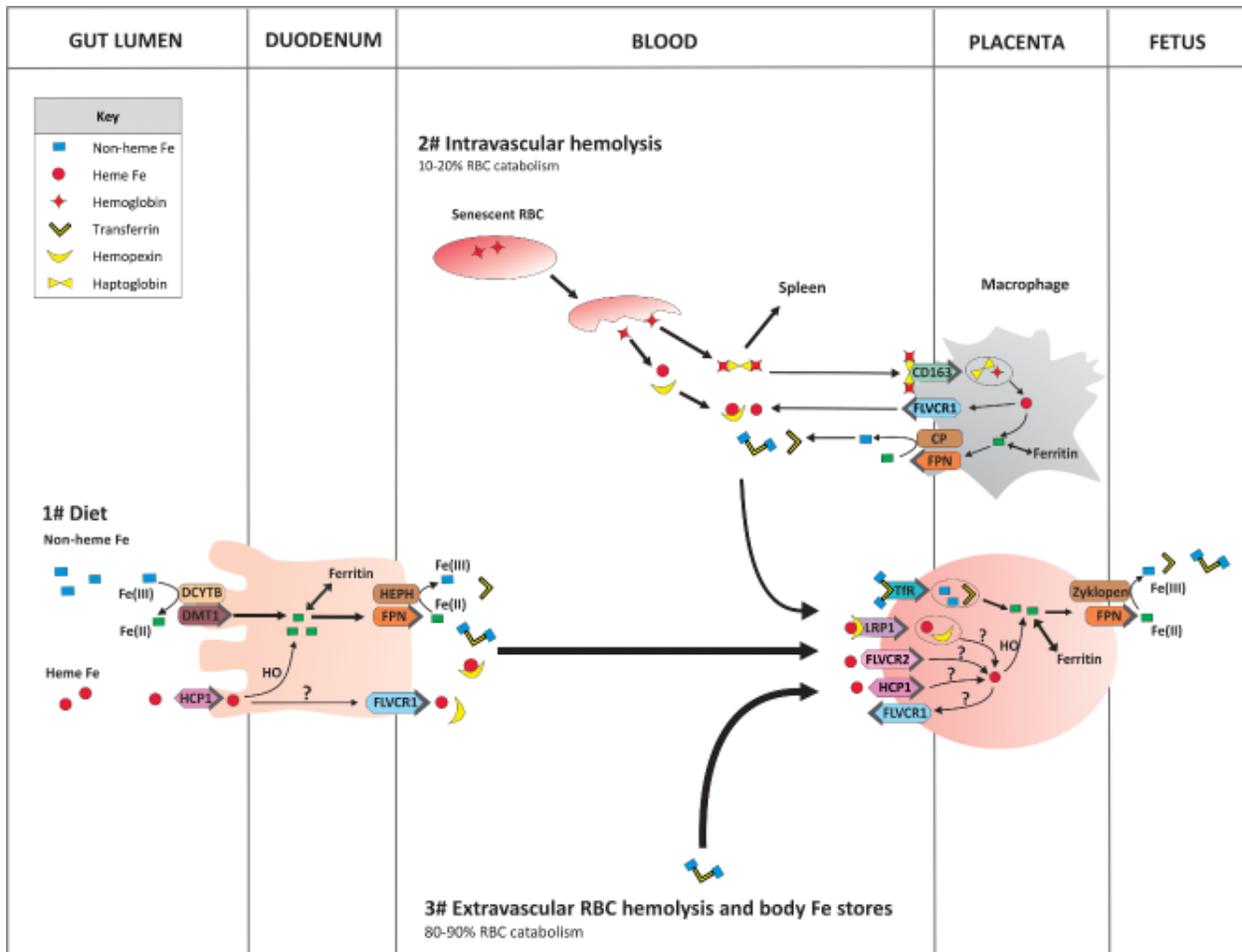


Figure 1.2. Fetal Fe sources and mechanisms of placental Fe transfer

Whether the presence of the heme transport and catabolic proteins in the human placenta indicate this organ can utilize dietary or maternal endogenous heme sources remains largely unknown. Consistent with the hypothesis of heme Fe utilization in the placenta, we have recently reported an inverse association between placental expression of the heme exporter FLVCR1 and maternal SF in teens with depleted Fe at delivery, suggesting that heme Fe may participate in placental Fe trafficking, especially when fetal Fe demands peak in third trimester of pregnancy (113). We have also recently found preferential fetal uptake of maternally ingested heme Fe

source compared to placental uptake of Fe from a dietary non-heme Fe source, which suggests there may be a preferential ability of the placenta to use dietary Fe of heme-origin (118).

Impact of other nutrients on Fe metabolism during pregnancy

Many nutrients can interact with Fe to influence Fe bioavailability at the level of the gut and/or at the systemic level to impact regulation of whole body Fe homeostasis. Dietary factors that are known to enhance non-heme Fe absorption include vitamin C (140), and animal tissues (141), while other dietary factors (such as phytates (142) and calcium (143)) inhibit non-heme Fe absorption. These associations that are often evident in single-meal studies are not always found when looking at relationships based on habitual dietary intakes. For example, while calcium intake can reduce non-heme Fe absorption if ingested in the same meal (143), calcium consumption does not appear to have a significant impact on Fe balance as indicated by several population surveys that found no association between dietary calcium intake and SF concentrations (145;146). Absorption of heme Fe, in contrast, appears to be little impacted by known dietary inhibitors or enhancers of non-heme Fe. There is some evidence that both meat (144), and soy proteins (145) increase the bioavailability of heme, possibly by preserving heme in the monomeric state (146).

Many processes involved in systemic Fe metabolism are dependent upon the status of other nutrients. Copper deficiency is thought to cause systemic ID and anemia by lowering the level and activity of two copper-containing ferroxidases (hephaestin and ceruloplasmin) which are essential for Fe export from the intestine (147), and from Fe stores (148). In rats, dietary copper deficiency during pregnancy was associated with reduced fetal Fe stores, suggesting a role for copper in placental Fe transport (149). This association may be mediated by the recently identified multi-copper ferrioxidase, Zyklopen, which is enriched in the placenta compared to

other tissues such as the heart and kidney (115). Zyklopen has also been observed to be up-regulated in response to cellular copper deficiency in human placental trophoblast-like BeWo cells (115).

Zinc supplementation trials in human adults have demonstrated adverse effects of dietary zinc on Fe homeostasis as evidenced by its ability to decrease Fe absorption (20), and by findings of lower serum Fe in zinc-supplemented pregnant women compared to the non- or Fe-supplemented groups (33). Although the mechanisms underlying these effects remain unclear, there is evidence for a direct stimulatory effect of zinc on intestinal expression of DMT1 and FPN (150), and for a common transport pathway in the liver possibly mediated by Zip14 (151).

Another micronutrient deficiency frequently associated with anemia during pregnancy is vitamin A deficiency (152). Vitamin A affects multiple aspects of Fe metabolism including absorption (156), RBC production, and mobilization from tissue Fe stores (153). Intervention studies in anemic pregnant women showed that concurrent supplementation of both vitamin A and Fe is more effective at improving Hb levels than either vitamin A or Fe alone, suggesting a synergistic relationship of these two nutrients in promoting erythropoiesis during pregnancy (154, 155).

Maternal ID and its consequences on the neonate

For many years, little attention was focused on the impact of maternal Fe status on neonatal Fe stores at birth since it was thought that the fetus functioned as a “perfect parasite” and would be able to acquire sufficient Fe even in the face of mild to moderate maternal anemia. Many new developments on regulation of Fe physiology and neonatal Fe status have challenged this assumption. An increasing body of literature supports the premise that fetal Fe needs will be compromised when maternal Fe stores are suboptimal (25, 114, 156, 157). There is also a

growing body of literature that suggests that altered or limited Fe supply *in utero*, during key windows of development, may lead to adaptive responses that permanently impact metabolic or developmental programming and the developing brain (158, 159). The timing at which ID occurs *in utero* has been found to impact subsequent outcomes in the offspring (160, 161). Suboptimal Fe stores at birth are associated with long-term, irreversible cognitive deficits in the offspring (162, 163). Of note, cord SF concentrations $< 76 \mu\text{g/L}$ have been associated with impaired language ability, tractability and fine motor skills in children subsequently studied at 5 years of age (164). The Fe endowment at birth is also critical in insuring that the neonate maintains Fe stores over early infancy because the neonatal gut is developmentally immature and may not regulate Fe absorption in response to Fe stores until 6-9 months of age (165, 166).

Greater attention on relationships between maternal Fe status and neonatal Fe stores is needed based on new findings including data indicating that infants whose mothers were anemic during pregnancy have been found to be more than 2-times as likely to have an abnormal laboratory indicator of Fe status at 9 months of age in comparison to infants born to non-anemic women (167). Assessment of Fe status in neonates is challenging as there are few normative data because universal screening for anemia is considered unwarranted (168), and venipuncture is not routinely undertaken among healthy newborns.

Assessing Fe status in newborns

Existing reference data define anemia in infants born after 34 weeks of gestation as a central venous Hb $< 13 \text{ g/dL}$ or a capillary Hb $< 14.5 \text{ g/dL}$ (169). Efforts to establish reference ranges for Hb in neonates have been hindered by the technical difficulty in obtaining venipuncture specimens (170), high variability in previously used measurement approaches (13, 171), and the small sample sizes of most reference value studies (172-174).

Using archived records of a large number of neonatal patients with minimal hematology-related pathologies, a series of recent publications sought to establish reference ranges (5th and 95th percentiles) for commonly measured hematologic parameters in preterm to term infants (13, 15, 175). With these data, the reference ranges of Hct and Hb for term neonates were set at 42-62% and 14-22 g/dL respectively, which are, on average, 10% and 3.3 g/dL higher than those for neonates born before 28 weeks of gestation (15). Additional challenges are evident in the assessment of neonatal Fe status because both Hb and Hct increase slightly during the first few hours after birth and then decline linearly such that by 28 days after birth values are lower than observed at birth (13). Because most hematologic parameters are impacted by postnatal age and also vary considerably with advancing gestation (171), both gestational-and-postnatal-age-specific reference ranges are needed to fully interpret population and individual data among newborns. An additional challenge in interpretation occurs because the mode of blood collection (e.g., venous, arterial, capillary) is not always recorded and can impact values obtained. Due to poor perfusion of a neonate's extremities, capillary Hb and Hct are about 5-25% higher than those obtained simultaneously from venous or arterial blood (171).

National data on neonatal Fe status does not exist as 1-2 year-olds are the youngest age group monitored for anemia prevalence by National Health and Nutrition Examination Survey. A recent report from American Academy of Pediatrics (AAP) emphasized the importance of screening infants and young children for ID due to its potential adverse effects on neuro-development (5). As noted by this report and others, no single screening test is available that will capture the full spectrum of Fe status in pediatric populations and measurements of Fe status in addition to Hb must be determined before ID is diagnosed (5). The AAP suggests two options for IDA screening: Hb < 11 g/dL and measurement of 1) SF and CRP, or 2) reticulocyte Hb

concentration. To identify ID without anemia, the use of SF, CRP, and reticulocyte Hb concentration is recommended (5). The usefulness of these measures in the assessment of Fe status during early infancy or at birth has not been fully evaluated. In addition, many core clinical laboratories do not have the instrumentation needed to measure reticulocyte hemoglobin concentration limiting the utility of this index as a measure of neonatal Fe stores. Normative ranges for many neonatal Fe status indicators that have been used in the literature are presented in **Table 1.2**.

Table 1.2. Normal ranges of hematological values in cord blood and capillary blood from term neonates¹

| | Cord blood | Capillary blood (1–3 days of age) |
|--|------------|--------------------------------------|
| Hb (g/dL) | 13.5–19.5 | 14.5–22.5 |
| Anemia cut-off | < 13.0 | < 14.5 |
| Hematocrit (%) | 42–60 | 45–67 |
| Erythrocyte count (10 ¹² /L) | 3.9–5.5 | 4.0–6.6 |
| Mean corpuscular volume (fL) | 98–118 | 95–121 |
| Mean corpuscular Hb (pg) | 31–37 | 31–37 |
| Mean corpuscular Hb concentration (g/dL) | 30–36 | 29–37 |
| SF (µg/L) | 40–309 | |
| Tissue Fe depletion | < 34 | |

¹ Cut-offs and ranges as published in Siddappa et al. (176), Brugnara (169), and Siddappa et al (14). All reference ranges are central 95% intervals of the reference populations except for SF, which consists of the central 90% of the reference population.

Cord clamping and neonatal Fe status

Delaying the time at which the umbilical cord is clamped post-delivery has a significant impact on the net amount of blood, and hence Fe stores, transferred to the neonate at birth. On average, delaying clamping until the cord has stopped pulsing translates to an additional 30% more blood volume in the neonate which would contribute up to 60% more RBC (177-179). Delayed cord clamping has also been linked to persistent benefits on early Fe homeostasis as evidenced by better Fe stores (ferritin) at 6 months of age (178, 180, 181). Concerns with this

practice have to do with a possible link with an increased risk of postpartum hemorrhage in the mother and neonatal jaundice in the newborn. A recent Cochrane review of this topic did not support any increased risk to the mother (on either postpartum or severe postpartum hemorrhage) but did find that delayed cord clamping resulted in significantly higher Hb levels in the neonate at birth and higher SF concentrations at 6 months (182). However, the need for early phototherapy for jaundice was slightly increased in those with late cord clamping (182).

Because cord clamping practices are not standardized and may be highly variable as a consequence of medical beliefs and birth/delivery circumstances, it is perhaps not surprising that data on relationships between maternal and neonatal Fe status are often conflicting.

Vulnerable maternal and neonatal groups

There are several high risk maternal groups for which gestational anemia screening or assessment of neonatal Fe status at birth may be warranted. Since most Fe is obtained during the third trimester of pregnancy, infants that are born preterm have a limited window of opportunity to accrue *in utero* Fe stores. Since maternal ID itself increases the risk of preterm birth, low birth weight infants that are born preterm may be particularly vulnerable. The AAP highlights both prematurity and low birth weight as risk factors for ID and IDA at 1 year of age (5). Additional risk factors for ID and IDA that were highlighted by the AAP include: exclusive breastfeeding beyond 4 months of age without supplemental Fe, weaning to whole milk diet low in Fe and among those with poor growth, low socioeconomic status and children with medical concerns that lead to feeding problems (5). Several other common pregnancy related complications or population groups may also be at increased risk for anemia over the course of pregnancy including; women with gestational diabetes mellitus, women carrying multiples, pregnant adolescents and women with inflammatory or infectious conditions across pregnancy.

Gestational diabetes

Gestational diabetes mellitus is a common problem among pregnant women affecting approximately 7% of pregnancies in the US (183). Gestational diabetes increases the risk of neonatal macrosomia, respiratory distress syndrome, and hypoglycemia and has also been linked to abnormal Fe acquisition by the developing fetus (184-188). Mechanisms responsible for the limited maternal-fetal Fe transfer are thought to involve a combination of chronic fetal hypoxemia and increased erythropoiesis leading to impaired Fe stores (156). Placental up-regulation of TfR occurs likely as a regulatory response to accommodate the increased fetal Fe demand (187), but this up-regulation may be insufficient to fully compensate for fetal Fe deficits because TfR has been found to be hyperglycosylated in diabetic pregnancies (185). When net Fe delivered to the developing fetus is limited, Fe may be prioritized to support fetal RBC production over other fetal tissues and Fe storage compartments. Net Fe content of the brain, liver and heart have been found to be decreased by 40-90% in autopsy studies of infants born to diabetic women when compared to control infants matched for gestational age at birth (188). The dysregulated maternal-fetal Fe homeostasis in diabetic pregnancies has been linked to functional consequences for the neonate/child as evidenced by impaired auditory recognition memory processing at birth (176), and altered explicit memory performance in children from diabetic pregnancies at 3.5 years of age (189).

Adolescent pregnancy

Recent figures from the Centers for Disease Control and Prevention (CDC) indicate that a total of 1,100 adolescents give birth each day in the US; 1 in 10 new mothers is an adolescent and teen childbearing costs US taxpayers more than 9 billion dollars per year (190). Few studies have specifically focused on pregnant adolescents to characterize the magnitude, determinants

and potential adverse health consequences associated with anemia in this age group. Minority adolescents may be particularly vulnerable as they are at higher risk for both early childbearing and anemia. Data from 1,141 African-American adolescents receiving care at a prenatal maternity clinic in Baltimore, Maryland found that while 9-13% of pregnant adolescents were anemic during early gestation, this prevalence increased to 57- 66% by the third trimester of pregnancy (191). Even after adjusting the normal Hb range downwards to 9.0-10.5 g/dL, to account for the 0.8 g/dL lower Hb observed among blacks (191), maternal anemia in adolescents was associated with a nearly 2-fold increased risk of low birth weight and preterm birth (10). In a longitudinal study of 80 minority teens, teens with a TfR/SF ratio > 300 in the second trimester were 12-times more likely to be classified with IDA during their third trimester of pregnancy, and 31% of teens had a TBI < 0 mg/kg in late gestation (12). While teens with low Fe reserves have been found to up-regulate placental TfR to meet fetal Fe demands, 9% of babies born to adolescent mothers were found to have ferritin stores $\leq 34 \mu\text{g/L}$ at birth (127). This may have functional consequences as ferritin concentrations $\leq 34 \mu\text{g/L}$ have been associated with brain ID, impaired auditory recognition memory, and lower psychomotor development scores (176).

Multiple births

Multiple births in the US are rapidly increasing in large part due to assisted reproductive technologies. From 1980-1999 alone, the rate of triplet births increased by more than 400% and twin births by more than 50% (192). Recent data indicate that multiple births now comprise 3-4.5% of all births in the US (193). Women carrying multiples are at increased risk for premature birth; nationally the average gestational age at delivery for those carrying twins is 37 weeks and for those carrying triplets it is 33 weeks (194). Preterm birth is a known risk factor for early anemia screening as emphasized by the AAP (5). Women carrying multiple fetuses may be at

even greater risk of ID due not only to the high Fe costs required by multiple fetal/placental units, but also due to their high gestational weight gains which could potentially increase systemic inflammatory mediators such as interleukin-6 (IL-6). The US currently has no specific nutritional recommendations for women carrying multiples, nor are there specific screening recommendations for their neonates to insure the Fe stores at birth are adequate. Obtaining normative data on Fe status in women carrying multiples will identify the degree to which Fe insufficiency is present in this group. Should anemia and/or low neonatal Fe stores be prevalent, subsequent screening and intervention approaches can be developed to target this high-risk group.

Conclusion

Many women enter pregnancy with insufficient Fe reserves and lack access to foods rich in Fe or Fe supplements. Recent reviews have highlighted the positive impact of maternal Fe supplementation on maternal birth outcomes, preterm birth and low birth weight but questions remain on how Fe supplementation influences maternal Fe stores at parturition, neonatal Fe status at birth and subsequent health outcomes in the child. In spite of the known associations between maternal anemia and adverse outcomes, more research is needed to design effective interventions to prevent or minimize maternal anemia. Additional work is needed to assess relative benefits of single vs. multivitamin mineral supplements on maternal status across pregnancy. To date, the majority of attention on Fe intake and pregnancy outcomes has focused on non-heme Fe. Heme Fe is independently regulated and has greater bioavailability in the mother. New data suggests there may be differential use of Fe from non-heme or heme sources during pregnancy. Similarly, while the placenta has some of the highest expression of heme

trafficking proteins of all tissues in the human body, the role, regulation and functions of these proteins in the placenta are poorly described in relation to maternal and neonatal Fe status.

To fully assess benefits and drawbacks to Fe supplementation, research and analytical approaches are needed to understand the physiological anemia of pregnancy that occurs due to hemodilution. In women with insufficient plasma volume expansion, more research is needed to identify biomarkers of this process in order to account for this measure in interpretation of circulating Fe status indicators. Additional work is also needed to identify the mechanisms responsible for hemoconcentration during pregnancy and the role of Fe intake in this process.

Hepcidin is now known to be a systemic Fe regulatory hormone, but questions remain on the role of this hormone on maternal, placental and neonatal Fe status. The fetus independently produces its own hepcidin early in gestation; the degree to which fetal hepcidin regulates placental Fe trafficking proteins in response to fetal Fe needs merits further attention. Because hepcidin explains only 20-30% of the variability in Fe absorption, additional data are needed to identify other genetic and environmental factors that impact maternal and neonatal Fe homeostasis.

Fe status *in utero* may program the fetus and impact brain development and subsequent neurobehavioral and cognitive outcomes. Animal studies have indicated that critical windows of development may exist across gestation and additional work is needed to explore if similar relationships exist in humans and if the timing and type of Fe supplementation given impacts outcomes in the offspring. Universal anemia screening is recommended in infants at 1 year of age but assessment at this late date may miss infants at increased risk for early ID. Given the explosion in multiple births, the continued problem of teen pregnancy, and the increase in risk of

obesity and gestational diabetes mellitus, these groups may benefit from additional neonatal screening to insure that Fe status in the neonate at birth is optimal.

Limited normative data are available on Fe status indicators among healthy neonates. Birth practices such as cord clamping can markedly impact Fe stores in the neonate and confound exploration of relationships between maternal and neonatal Fe status. The impact of neonatal Fe status at birth on acute and long-term functional outcomes is a growing area of interest. Many new approaches are available to assess functional outcomes in the neonate; continued application of these approaches among at-risk cohorts will allow for better targeting and intervention of neonates with suboptimal Fe reserves at birth.

The 1997 review by Allen (122) on pregnancy and ID ended with the statement: “It is possible that efforts to improve the situation, and compliance by mothers, might be improved if adverse impacts of ID during pregnancy were better documented. Conversely, if the impacts are minimal, it may be more appropriate on a global scale to focus on severe anemia and to view pregnancy as a situation of temporary anemia and iron depletion that does not require intervention in every woman.” Many advances have been made over the past 15 years of research on this topic yet many unresolved issues remain. The impact of Fe supplementation during pregnancy extends beyond the pregnant women to her developing fetus. Additional focus on the role of Fe in promoting optimal maternal and neonatal outcomes is needed at these key life stages.

Research Overview and Specific Aims

Data from National Health and Nutrition Examination Survey indicate that the prevalence of iron deficiency (ID) among children age 1-2 years has more than doubled over the past decade, reaching 15.9% in 2008 (195). ID during infancy delays the development of the central nervous system and results in cognitive, social-affective, and psychomotor impairments. At present, there are no nutritional screening programs for ID in infants < 12 months (196). In addition, the regulatory capacity of the enterocyte may not be fully developed during early infancy and may not adequately modify iron (Fe) absorption in response to Fe status (197). For this reason low Fe stores at birth are a major risk factor for infant ID (198). A better characterization of the major factors involved in the establishment of neonatal Fe stores will help formulate effective prenatal strategies to maximize the birth Fe endowment and minimize adverse effects of postnatal Fe insufficiency.

Fe in the diet exists in two forms: non-heme Fe and heme Fe. Heme Fe only represents 10% of total dietary Fe intake but accounts for one-third of absorbed Fe due to its high bioavailability (199). In contrast to the relatively well-defined mechanisms of non-heme Fe absorption, the specifics of heme Fe absorption remain uncertain. Over the past decade, several heme Fe transporters have been identified in the intestine yet their role in Fe absorption remains unexplored. Despite extensive evidence for the importance of maternal diet in insuring adequate fetal Fe supply, little is known about endogenous maternal Fe sources and their contributions to fetal Fe. It is well recognized that dietary Fe alone is not enough to meet the high fetal demands during the third trimester of pregnancy and nearly 30% of women enter pregnancy with no Fe stores (6). Endogenous Fe obtained from the degradation of senescent RBCs constitutes the

largest single Fe influx into the circulation (20 mg Fe/day) (200), and may represent a potentially important Fe source of heme and non-heme iron for the fetus. However, virtually no information is available on the utilization of this Fe source during pregnancy. The majority of Fe derived from RBC breakdown is recycled back into maternal circulation as non-heme Fe but a small portion is thought to be released as heme or hemoglobin (Hb) (139). The human placenta contains all known receptors and catabolic enzymes involved in heme Fe utilization (201). At present, questions remain as to whether any of these proteins play a role in placental Fe transfer and how they are regulated in response to maternal and fetal signals. Recent data in rats highlights the ability of the fetus to regulate placental Fe transfer via the action of the fetal systemic Fe regulatory hormone, hepcidin (121). This hypothesis has yet to be validated in humans. Furthermore, while the fetus may have some capacity to regulate Fe transfer, the relative importance of maternal versus fetal hepcidin in regulating placental Fe transfer remains to be determined.

To address gaps in intestinal and placental Fe transport and the regulation of Fe homeostasis during pregnancy, we undertook a series of animal and human studies with the following five specific aims:

Aim 1: To review literature on Fe utilization during pregnancy

Current status: A review on various aspects of Fe related topics in pregnancy including Fe supplementation, mechanisms of placental Fe transport, assessment of maternal and neonatal Fe status was published in *Nutrition Reviews* in 2013. The review is included in **Chapter 1** as the background for this doctoral work.

Aim 2: To determine the impact of hepcidin on intestinal heme Fe absorption in rats with induced Fe overload

Hypothesis: 1) Fe loading in rats will increase liver hepcidin production and strongly suppress duodenal expression of non-heme Fe transporters and non-heme Fe absorption; and 2) Heme Fe absorption and duodenal heme Fe transporters will be less impacted by hepcidin up-regulation.

Current status: The manuscript is presented in **Chapter 2** and is undergoing second round of review at the *Journal of Nutrition* as of July 2014.

Aim 3: To measure placental expression of heme Fe transporters and identify the determinants of their expression

Hypothesis: Placental expression of two heme transporters (LRP1 and PCFT) will be associated with maternal and neonatal Fe status and the Fe regulatory hormone, hepcidin.

Current status: The manuscript on placental LRP1 expression is in press at *Reproduction*. The manuscript on placental PCFT expression has been written and will be submitted to the *Journal of Nutrition* by the end of July. Both manuscripts are presented in **Chapter 3**.

Aim 4: To assess the relative contributions of maternal RBC catabolism and dietary Fe absorption to fetal Fe stores in human pregnancy

Hypothesis: 1) A greater fraction of Fe derived from maternal RBCs will be transported to the fetus when compared to relative amounts of Fe transferred to the fetus from maternal diet during the third trimester of pregnancy; and 2) Placental transfer of both maternal Fe sources will be negatively regulated by neonatal hepcidin.

Current status: A total of 16 participants have been recruited since August, 2012 and 12 women have completed the study. Sample collection from all participants will be finished in October 2014, laboratory analysis will be completed by December 2014, and the manuscript will be submitted for review by January 2015. Preliminary results are presented in **Chapter 4**.

Aim 5: To determine the impact of pre-pregnancy obesity and excessive gestational weight gain on maternal hepcidin and neonatal Fe status in pregnant adolescents

Hypothesis: Maternal obesity and excessive gestational weight gain will be associated with elevated inflammation as measured by interleukin 6 and hepcidin and this will be associated with deficits in neonatal Fe status at birth.

Current status: The manuscript is presented in **Chapter 5** and is currently under review at the *Journal of International of Obesity* as of July 2014.

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CHAPTER 2

DUODENAL ABSORPTION AND TISSUE UTILIZATION OF DIETARY HEME AND NON-HEME IRON IN A RAT MODEL OF IRON OVERLOAD²

Abstract

Dietary heme contributes to Fe intake yet regulation of heme absorption and tissue utilization of absorbed heme remain undefined. In a rat model of Fe overload, we utilized stable Fe isotopes to examine heme and non-heme Fe absorption in relation to liver hepcidin and to compare relative utilization of absorbed heme and non-heme Fe by erythroid (RBC) and Fe storage tissues (liver and spleen). Twelve male Sprague Dawley rats were randomized to injections of either saline or Fe dextran (16 mg or 48 mg of Fe over 2 weeks). After Fe loading, rats received oral stable Fe in the forms of ⁵⁷Fe-ferrous sulfate and ⁵⁸Fe-labeled hemoglobin. Expression of liver hepcidin and duodenal Fe transporters and tissue stable Fe enrichment were determined 10 days post-dosing. High Fe loading increased hepatic hepcidin by 3-fold and reduced duodenal expression of divalent metal transporter1 by 76%. Non-heme absorption was 2.5-times higher than heme Fe absorption. Absorption of both forms of Fe were inversely correlated with hepatic hepcidin expression but hepcidin had a stronger impact on non-heme Fe absorption (p=0.04). Significantly more ⁵⁷Fe was recovered in RBC and more ⁵⁸Fe was recovered in the spleen. Elevated hepcidin significantly decreased heme and non-heme Fe absorption but had a greater impact on non-heme Fe absorption. Differential tissue utilization of heme vs. non-heme Fe was evident between erythroid and Fe storage tissues, suggesting some heme may be exported into the circulation in a different form than non-heme Fe.

² Cao C, Thomas CE, Insogna KL, O'Brien KO. In revision at *Journal of Nutrition* July 2014.

Introduction

Fe is an essential micronutrient for humans and plays a critical role in a variety of processes such as oxygen transport and energy production. Because humans have no regulated mechanism of Fe excretion, Fe balance relies primarily on tight regulation at the level of intestinal Fe absorption (1). Dietary Fe is present in two distinct forms: heme Fe (animal products) and non-heme Fe (plants, animal products and supplements). The absorption of non-heme Fe is tightly controlled by body Fe status and the liver-derived peptide hormone, hepcidin, the hormone that responds to body Fe reserves to regulate Fe homeostasis (2). Hepcidin is thought to affect Fe absorption by inducing the degradation of the basolateral Fe transporter ferroportin (FPN), thus limiting Fe export into the circulation (3). However, this classic model of hepcidin action has been challenged by accumulating cell culture and animal data showing mixed results regarding the relationship between hepcidin and duodenal FPN (4-7). Recent studies in mice and Caco-2 cells further suggest that the brush boarder Fe transporter, divalent metal transporter 1 (DMT1), not FPN, is the primary target of hepcidin regulation (5). Clearly, *in vivo* experiments that explore the interrelationships between hepcidin, intestinal Fe transporter expression, and non-heme Fe transport are needed to clarify the complex mechanisms by which hepcidin modulates absorption of dietary heme and non-heme sources of Fe.

Heme Fe constitutes ~10% of total dietary Fe intake in a typical western diet but is thought to provide nearly one-third of absorbed Fe because of its substantially higher absorption and bioavailability (8). Despite the importance of heme in Fe nutrition, the mechanisms responsible for heme Fe absorption remain largely uncharacterized. In contrast to non-heme Fe, heme Fe absorption is less stringently regulated in response to Fe status and dietary inhibitors and enhancers. Apical heme uptake is thought to occur either via receptor mediated endocytosis

(9), or may in part be mediated by a low affinity heme importer, proton coupled folate transporter (PCFT) (10), or to an as yet identified heme importer. It is believed that once in the enterocyte, the majority of heme Fe is converted to inorganic Fe and enters a common intracellular Fe pool with non-heme Fe to be exported out to the circulation by FPN. An alternative heme enterocyte export pathway has been described in guinea pigs whereby heme is exported intact into the plasma (11). The presence of the heme exporter, feline leukemia virus C receptor 1 (FLVCR1), in the human duodenum supports this hypothesis (12). Little is known about the relative impact of hepcidin on heme versus non-heme Fe absorption and the degree to which Fe overload differentially influences duodenal and tissue expression of heme and non-heme Fe transporters.

The goal of this study was to utilize a rat model of Fe overload and labeled heme and non-heme Fe to examine the impact of hepcidin on heme vs. non-heme Fe transport across the enterocyte in vivo and to evaluate associations between Fe loading and hepcidin expression on duodenal, hepatic and splenic expression of proteins involved in the transport of heme [PCFT, heme oxygenase (HO)-1] and non-heme Fe [DMT1, FPN, duodenal cytochrome b (Dcytb)]. We hypothesized that Fe loading would elevate liver hepcidin production and result in parallel reductions in duodenal FPN and non-heme Fe absorption and that Fe loading would have a substantially larger ability to down-regulate non-heme Fe trafficking proteins and absorption. We further hypothesized that heme and non-heme Fe absorption would be differentially impacted by hepcidin expression.

Methods

Animals and treatments

Twelve adult male Sprague Dawley rats (8-10 weeks of age) were maintained on a standard rodent diet (Fe content: 36 ppm; AIN93-G, Harlan Teklad, Indianapolis, IN) for a week before being randomized to one of three groups: high Fe loading, moderate Fe loading, or control group. Animals in the high and moderate Fe groups received a total of 48 mg and 16 mg Fe administered through intra-peritoneal injections of Fe dextran twice a week for two weeks from Day 0 to Day 13. A relatively mild regimen of Fe overload was chosen (~0.03 and 0.1 mg Fe/g body weight) when compared to other Fe loading rodent protocols (0.2-1.5 mg/g body weight) (13-16) to better characterize relationships between Fe absorption and hepcidin under more physiological conditions. Animals in the control group were injected with phosphate buffered saline (PBS) at the same time points during the same period. All study procedures were approved by the Yale Institutional Animal Care and Use Committee.

Isotope preparation

Stable Fe isotopes (^{57}Fe at 95% enrichment and ^{58}Fe at 93% enrichment) were purchased from Trace Sciences International (Richmond Hill, Canada). The ^{57}Fe isotope was converted into ferrous sulfate solution by Anazao Health Corporation (Tampa, FL) using the method of Kastenmayer et al (17). The ^{58}Fe isotope was converted into ferrous citrate and injected into a piglet to intrinsically label porcine RBC as described elsewhere (18). The ^{58}Fe enrichment of the labeled porcine RBC was $17.3 \pm 0.01\%$ as determined by magnetic sector thermal ionization mass spectrometer (ThermoQuest Corp., Bremen, Germany).

Iron absorption studies

Absorption of heme and non-heme Fe were measured 2-weeks after Fe loading using orally administered stable Fe isotopes. Oral stable Fe tracers containing similar amounts of total Fe (25.4 μg ^{58}Fe as porcine ^{58}Fe -Hb, total Fe from the Hb dose: 145.0 μg ; 172.1 μg ^{57}Fe as $^{57}\text{FeSO}_4$; total Fe from the FeSO_4 dose: 181.0 μg) were administered by oral gavage on two separate days using a randomized cross-over design so that half of the rats received the heme Fe tracer on Day 14 and non-heme Fe tracer on Day 17 and the remaining rats received these two forms of Fe in the reverse order. Fe tracer doses were separated by 3 days to allow sufficient time for the intestinal epithelium to renew in order to minimize any impact of the first tracer dose on absorption of the second tracer dose. Absorption of the heme and non-heme tracers was assessed by RBC enrichment 10 days after the administration of the second tracer since prior Fe tracer studies in rats have established that RBC incorporation of injected Fe is complete within 6 days of administration (19, 20). Rats were sacrificed 10-days post-dosing, and whole blood was collected by cardiac puncture. Serum was separated and stored at -80°C . Liver, kidney, spleen, heart, and duodenal tissue were harvested, flash frozen in liquid nitrogen, and stored at -80°C prior to analysis for Fe isotopic enrichment by TIMS as detailed below.

Blood and tissue Fe isotopic enrichment measurement

Samples of whole blood, liver, and spleen were digested with 4 mL HNO_3 in polytetrafluoroethylene beakers and evaporated to dryness after the solutions were clear. Samples were dissolved in 2 mL 6N HCl and Fe was extracted by anion exchange chromatography as described previously (21). The eluate was dried and reconstituted in 50 μl 3% HNO_3 . Extracted Fe samples (8 μL) were loaded onto rhenium filaments (H Cross Co, Weehawken, NY) with 4 μL silica gel (Sigma-Aldrich, St Louis, MO) and 4 μL phosphoric acid.

Isotopic ratios ($^{57/56}\text{Fe}$ and $^{58/56}\text{Fe}$) were measured using TIMS. The natural abundance values used were 0.02317 for $^{57/56}\text{Fe}$ and 0.00308 for $^{58/56}\text{Fe}$. Relative SDs averaged 0.025% and 0.30% for $^{57/56}\text{Fe}$ and $^{58/56}\text{Fe}$, respectively. All acids used were ultrapure (Ultrex; JT Baker, Phillipsburg, NJ).

Calculation of tissue isotope incorporation

The net amount of ^{57}Fe and ^{58}Fe incorporated in RBC was determined by multiplying RBC enrichment of the two tracers by the total RBC Fe mass assuming a blood volume for male Sprague Dawley rats of 68.6 mL/kg (22), and an Fe content of Hb of 3.47 g/kg (23). A similar approach was used to calculate the net amount of ^{57}Fe and ^{58}Fe in liver and spleen using the measured tissue ^{57}Fe and ^{58}Fe enrichment, tissue Fe concentration, and an estimated tissue weight for male Sprague Dawley rats derived from equations developed based on the age of the animal (24).

Calculation of Fe absorption

Fe absorption was calculated as the total amount of tracer recovered in RBC, liver and spleen as a fraction of the oral dose. Due to inadequate liver sample and nickel contamination in some spleen and liver samples which interfered with ^{58}Fe enrichment measures, data on total recovered ^{57}Fe and ^{58}Fe were only available for 11 and 9 of the 12 rats, respectively. From the animals with complete tissue enrichment data, we determined that 80.5% and 71.3% of the total recovered ^{57}Fe and ^{58}Fe were found in RBC. This allowed us to make assumptions about RBC incorporation of heme and non-heme Fe tracers for the 3 rats that did not have complete tissue enrichment data. We also calculated absorption based on 80% RBC incorporation frequently used in human studies to estimate heme and non-heme Fe absorption. This method did not change the relationships between heme/non-heme Fe absorption with hepcidin.

Calculation of Fe absorption and tissue Fe uptake

The oral Fe tracer has two primary fates once absorbed across the enterocyte: it is either utilized by RBC for Hb synthesis or delivered to storage sites (primarily the liver and spleen) (Figure 2.1). Absorption of heme and non-heme Fe was calculated as the total amount of heme (^{58}Fe) or non-heme (^{57}Fe) tracer recovered in RBC, liver, and spleen as a fraction of the total oral tracer dose administered. Tissue utilization of absorbed tracers was assessed using 3 approaches. The first approach presented the delta percent excess of each isotope, which reflects the degree to which the natural abundance of isotope in each tissue was increased as a result of tissue tracer uptake. The second approach estimated the net quantity (μg) of each Fe tracer recovered in the RBC pool, liver, and spleen. The third measure assessed the relative distribution of absorbed tracer in RBC, liver, and spleen by expressing the amount of tracer in each tissue as a proportion of the total tracer recovered.

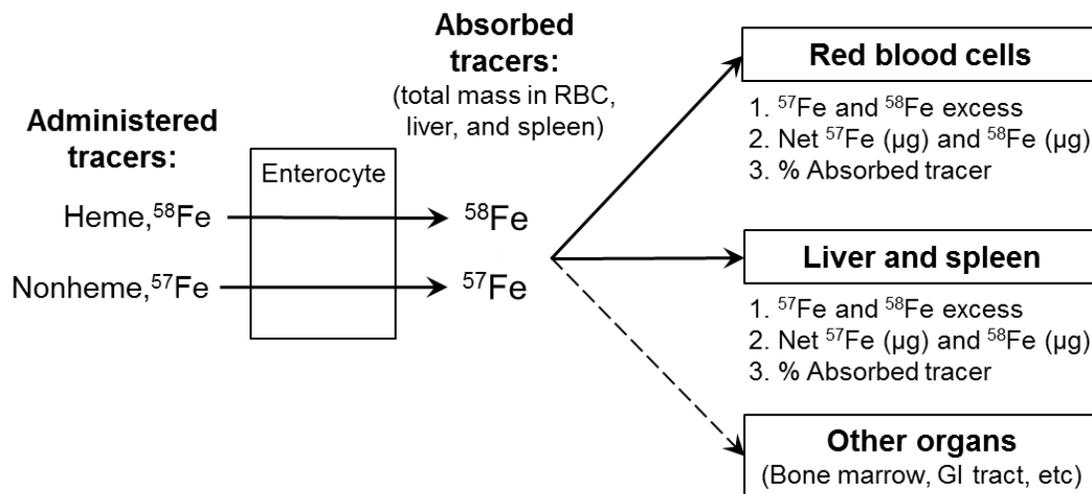


Figure 2.1. Calculation of duodenal absorption and tissue distribution of dietary heme and non-heme Fe isotopes

Tissue Fe determination

Tissue Fe concentration was measured using a graphite atomic absorption spectrophotometer (Perkin-Elmer 370, Norwalk, CT). Bovine liver standard 1577 (National Bureau of Standards) was analyzed in the same manner to insure accuracy of the Fe measurements.

Western blotting of tissue Fe proteins and serum ferritin

Protein was extracted from liver, spleen, and duodenum by homogenizing tissues in lysis buffer containing protease inhibitors (Sigma Aldrich, St. Louis, MO). Proteins (30 μ g) were solubilized in Laemmli buffer and boiled for 5 min at 95°C before SDS-PAGE and subsequent transfer onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in Odyssey Blocking Buffer (Li-Cor, Lincoln, NB) for 1 hour and incubated with primary antibodies overnight at 4°C. Antibodies and the concentrations used were as follows: anti-FPN (MTP11-A, Alpha Diagnostics, San Antonio, TX) at 1:1000 dilution; anti-TfR1 (No. 136800, Life Technologies, Grand Island, NY) at 1:5000 dilution; anti-PCFT (ab25134, Abcam, Boston, MA) at 1:1000 dilution; anti-HO1 (SPA-869, Enzo Life sciences, Farmingdale, NY) at 1:500 dilution; anti-ferritin (ab55077, Abcam, Boston, MA) at 1:1000 dilution. After incubation with the primary antibodies overnight at 4°C, membranes were washed five times and then incubated with appropriate infrared secondary antibodies (1:5000 dilution; Li-Cor, Lincoln, NE) at room temperature for 1 hr. Blots were visualized using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE) and the expression of target proteins were normalized to β -actin (sc47778, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and expressed as a ratio relative to the control group. Six duodenal lysates from six rats appeared to be partially degraded as indicated by substantially lower β -actin levels compared to other analyzed duodenal lysates. These lysates were excluded

from western blot analysis of duodenal Fe transporters. The degradation likely reflects the known presence of high concentrations of proteases in duodenal tissue.

Serum ferritin was analyzed by western blotting in serum samples collected on the day of sacrifice. Serum (5 μ L) was diluted with DI H₂O, separated on a 12.5% SDS-PAGE gel, and transferred to a PVDF membrane. After blocking, the membrane was incubated with a ferritin antibody (ab55077, Abcam, Boston, MA; 1:1000), probed with an infrared secondary antibody, and analyzed using the Odyssey imaging system as described above.

Quantitative reverse-transcription polymerase chain reaction (qPCR)

Total RNA was extracted from duodenal enterocytes and liver using TRIzol reagent (Invitrogen, Carlsbad, CA), followed by purification using the RNeasy Mini Kit (Qiagen, Valencia, CA). The cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA) and reversed transcribed using a PTC-100 PCR machine (MJ Research, Inc., Waltham, MA). qPCR was performed using TaqMan assays for the following targets: *Dmt1* (Rn00565927_m1), *Dcytb* (Rn01484657_m1), *TfR1* (Rn01474701_m1), *hepcidin* (Rn00584987_m1), *Pcft* (Rn01471182_m1), *hephaestin* (Rn00515970_m1), and *Fpn* (Rn00591187_m1) (Applied Biosystems, Foster City, CA). β -*actin* was used as the endogenous reference (Rn00667869_m1; Applied Biosystems). The PCR reactions were run using an iQ2 Optical System (Bio-Rad, Hercules, CA) with the following protocol: 95°C for 10 min, 40 cycles at 95°C for 20 sec, and 60°C for 1 min. Relative quantification of target genes was calculated using the comparative C_T method.

Statistical analysis

Statistical analyses were performed in JMP 10.0 (SAS, Cary, NC). Differences between treatment groups were compared using one-way ANOVA and Tukey's post-hoc test. Differences

in isotopic enrichment between liver, spleen, and blood were analyzed in a regression model coding rat ID as a random effect. Heme and non-heme Fe absorption were compared by paired t test. Simple linear regression was used to examine relationships between hepcidin, RBC Fe incorporation, Fe transporter expression, and tissue isotopic enrichment. An interaction term between Fe form (heme vs. non-heme) \times hepcidin was used to test whether the effect of hepcidin on Fe absorption differed between heme versus non-heme Fe. Data are expressed as Mean \pm SEM unless otherwise indicated. Statistical significance was defined when $P < 0.05$.

Results

Impact of Fe loading on tissue Fe content, hepcidin, and tissue Fe transporter expression

Two weeks of Fe loading with a total of 16 mg or 48 mg did not significantly influence body weight or Hb levels between the Fe loaded and control groups. Rats in the high Fe group had elevated tissue Fe concentrations (mg/g dry weight) in liver and spleen while those in the moderate Fe group only exhibited significant increases in the Fe content of the spleen. Fe loading significantly increased liver *hepcidin* mRNA in the high Fe group (**Figure 2.2**); the increase in the moderate Fe group did not reach significance ($p = 0.2$). Hepcidin correlated in logarithmic proportion to liver Fe concentrations ($r = 0.95$, $p < 0.0001$). Serum ferritin did not differ between the 3 treatment groups ($p = 0.1$) but was significantly higher in the combined high and moderate Fe group when compared to the controls ($p = 0.03$) (**Figure 2.2**).

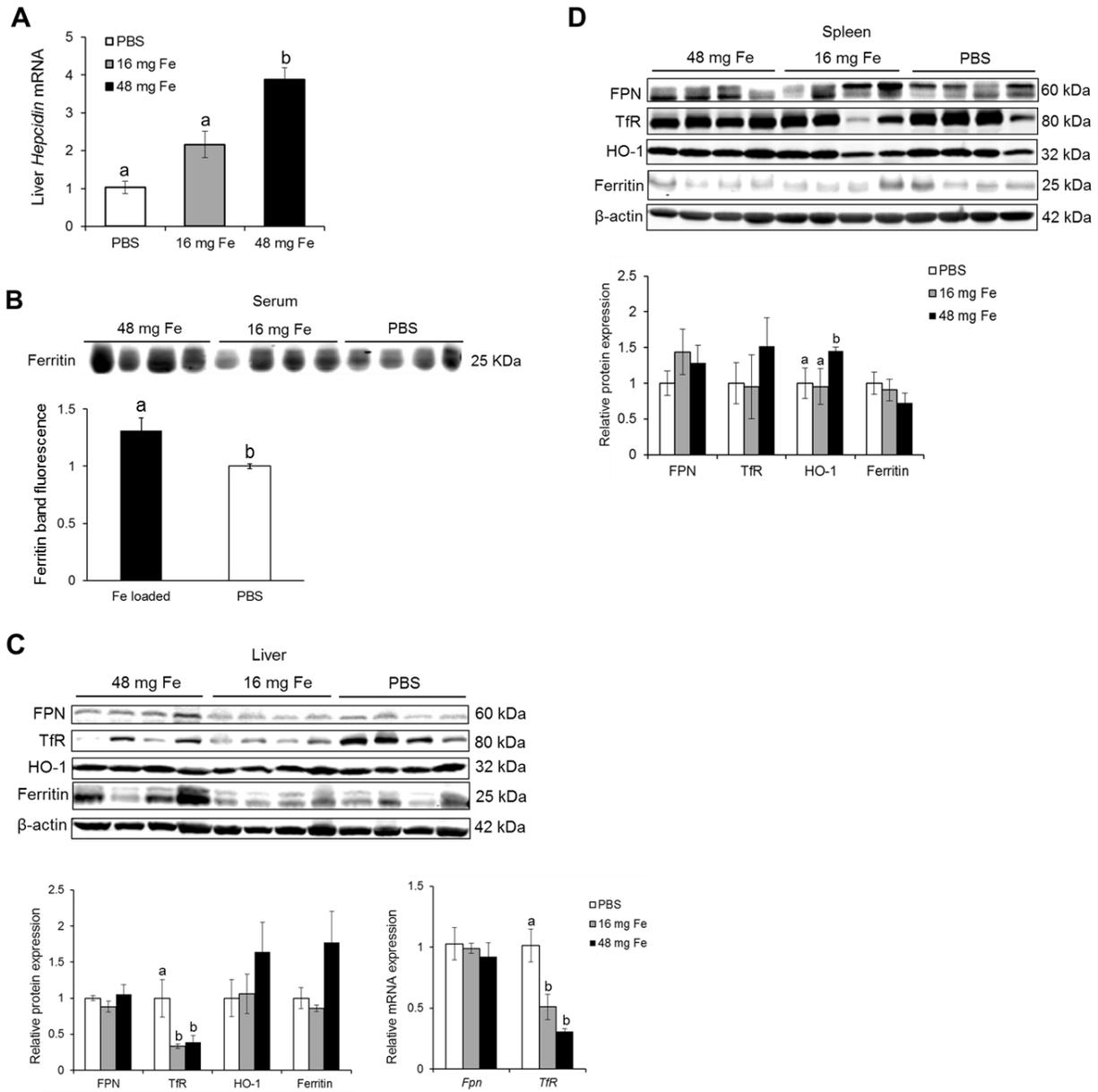


Figure 2.2. Effect of Fe injections on hepcidin and tissue Fe transporter expression in rats. (A) Liver *hepcidin* mRNA in rats injected with either PBS, 16 mg or 48 mg of Fe. Values represent mean \pm SEM (n=4). Bars with different letters are significantly different. (B) Serum ferritin protein expression determined by western blotting. (C) Liver expression of genes involved in Fe metabolism. (D) Expression of proteins involved in Fe metabolism in the spleen.

Expression of genes involved in Fe metabolism in the liver was determined by qPCR (*Fpn* and *TfR1*) and western blotting (FPN, TfR1, HO-1, and ferritin) (**Figure 2.2**). Fe injections significantly suppressed hepatic *TfR1* but had no impact on hepatic *Fpn* mRNA. Similar

treatment responses were noted for hepatic protein expression of TfR1 and FPN. Hepatic protein expression of ferritin and HO-1 in the high Fe group tended to be higher than the moderate and control groups but differences observed were not statistically significant. Expression of Fe proteins in the spleen was assessed by western blotting (**Figure 2.2**). Only HO-1 was significantly increased in response to moderate and high Fe injections. Together, these results indicate the high Fe dextran treatment produced the classic phenotype of Fe overload while the moderate Fe regimen only mildly elevated Fe stores in the spleen without significantly affecting hepcidin production or expression of tissue Fe transporters.

Impact of Fe loading on Fe absorption and duodenal Fe transporter expression

Average absorption efficiencies of heme and non-heme Fe in the control animals averaged $19.8 \pm 3.3\%$ (mean \pm SD) and $45.2 \pm 12.1\%$ (mean \pm SD), respectively. Heme and non-heme Fe absorption were strongly correlated within animals ($r = 0.79$, $p = 0.002$). In contrast to the significantly higher heme vs. non-heme Fe absorption that is known to occur in humans, the mean heme Fe absorption observed in all rats ($n=12$) was 60% lower than observed for non-heme Fe absorption ($n=12$, $p = 0.0007$). Injections of moderate and high doses of Fe dextran suppressed non-heme Fe absorption by 46% (24.1% vs. 45.2% , $p = 0.01$) and 78% (10.0% vs. 45.2% , $p = 0.0004$), respectively when compared to the control group. Percent heme Fe absorption was similarly decreased in the moderate (7.5% vs. 19.8% , $p = 0.0002$) and high Fe group (4.7% vs. 19.8% , $p < 0.0001$) when compared to the control group (**Figure 2.3**). Although the degree of suppression of heme and non-heme Fe absorption was greater with the 48 mg load as compared to the 16 mg load, these differences did not reach statistical significance ($p = 0.08$ for non-heme Fe and $p = 0.3$ for heme Fe absorption).

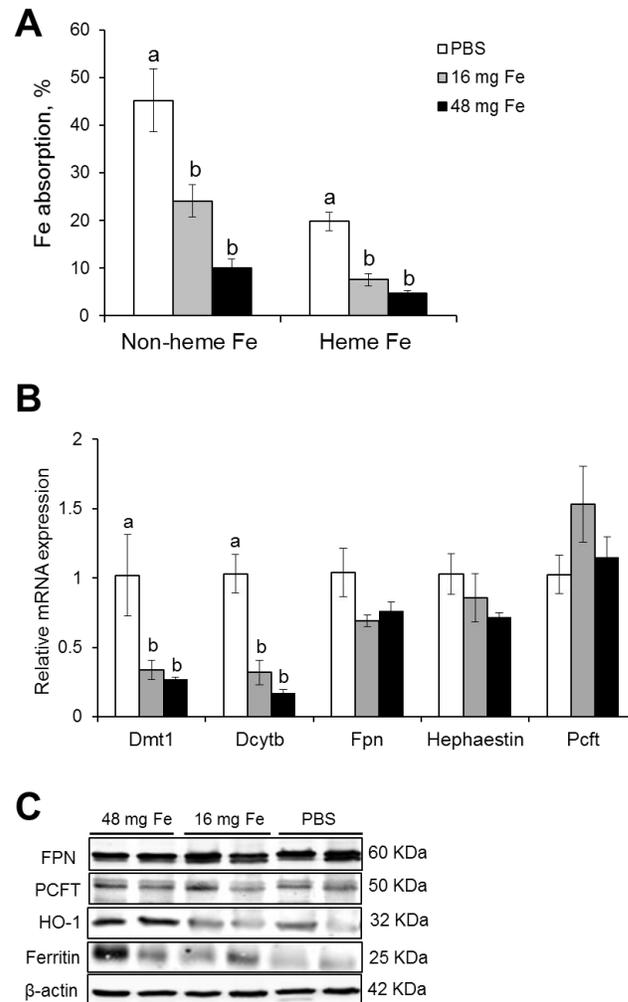


Figure 2.3. Fe absorption and duodenal Fe transporter expression in rats after Fe loading treatment. (A) Heme and non-heme Fe absorption expressed as total heme and non-heme Fe isotopes recovered in RBC, liver, and spleen as a percentage of oral doses administered. (B) Duodenal mRNA expression of genes involved in Fe transport relative to the control group. Bars without a shared letter are significantly different. (C) Western blotting of genes involved in duodenal Fe transport (n = 2/group).

Next we examined duodenal expression of genes involved in heme and non-heme Fe trafficking (*Dmt1*, *Dcytb*, *Fpn*, *hephaestin*, *Pcft*) and explored possible associations with heme and non-heme Fe absorption. Transcript levels of *Dmt1* and *Dcytb* decreased significantly in the high Fe group (**Figure 2.3**) and both were significantly negatively correlated with heme and non-

heme Fe absorption. However, no significant differences in mRNA expression of *Fpn*, *hephaestin*, or *Pcft* were evident in Fe loaded rats when compared to the control group. We also performed western blotting on selected heme and non-heme Fe proteins (FPN, PCFT HO-1, and ferritin) in duodenal lysates from 6 animals (**Figure 2.3**). Consistent with the mRNA results, there were no significant differences in FPN and PCFT protein expression between Fe loaded and control rats. Although only analyzed in two samples the expression of HO-1 and ferritin was higher in the high Fe group in both cases.

Effect of hepcidin on Fe absorption and tissue Fe transporter expression

Linear regression analysis showed that liver *hepcidin* mRNA correlated inversely with absorption of both heme ($r = -0.77$, $p = 0.003$, $n = 12$) and non-heme Fe ($r = -0.80$, $p = 0.002$, $n = 12$). The effect of hepcidin on Fe absorption differed significantly between the heme and non-heme Fe tracers with every unit increase in hepcidin expression reducing heme absorption by 4.1% and non-heme absorption by 10.0% ($p = 0.04$). The interaction between dietary Fe (non-heme vs. heme Fe) and hepcidin is shown in **Figure 2.4**.

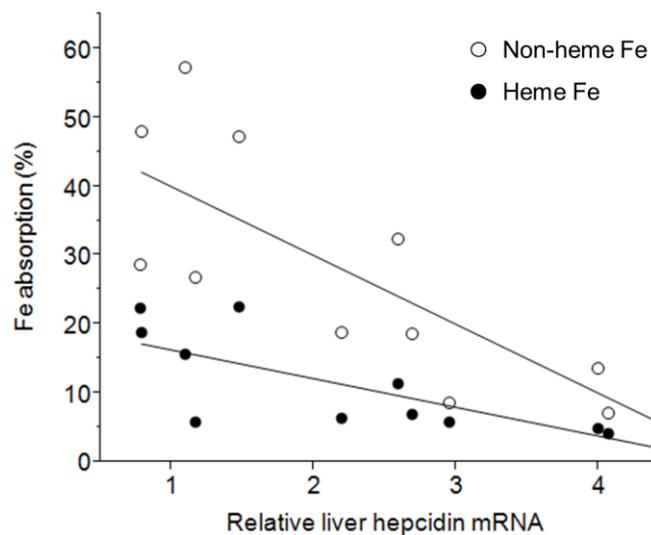


Figure 2.4. Correlations of liver hepcidin mRNA with heme and non-heme Fe absorption

To investigate the molecular basis for the observed difference in the impact of hepcidin on heme and non-heme Fe absorption, duodenal heme and non-heme Fe transporter expression was examined in relation to liver *hepcidin* transcript expression. Hepcidin was inversely associated with *Dmt1* ($r = -0.61$, $p = 0.04$) and *Dcytb* mRNA ($r = -0.72$, $p = 0.009$). There was a non-significant negative correlation between hepcidin and *Fpn* mRNA ($p = 0.13$). Hepcidin transcript expression did not correlate with duodenal *Pcft* mRNA expression. In the 6 rats with evaluable duodenal lysates, hepcidin protein levels were not associated with any of the Fe proteins examined (FPN, HO-1, PCFT, ferritin) except for a correlation with ferritin, which was not significant ($r = 0.68$, $p = 0.09$).

Tissue utilization of ingested heme and non-heme Fe

To explore whether there were potential differences in tissue utilization of absorbed heme and non-heme Fe, the relative recovery of the two tracers in RBC, liver, and spleen was evaluated (**Table 2.1**). A significantly higher percentage of non-heme Fe tracer was recovered in the RBC mass than that recovered for heme Fe (80.5% vs. 71.3%, $p = 0.02$). A reverse relationship was evident in the spleen, such that significantly more heme Fe tracer was recovered compared to the relative recovery of the non-heme Fe tracer (7.5% vs. 2.7%; $p = 0.01$). No significant difference in relative recovery of each form of Fe was noted in the liver. There was also a differential effect of hepcidin on tissue utilization of absorbed heme and non-heme Fe. In the RBC pool, the net amount of both ^{57}Fe and ^{58}Fe tracer recovered was inversely correlated with liver hepcidin ($r = -0.75$, $p = 0.005$; $r = -0.76$, $p = 0.005$, respectively). Hepcidin was not related to the net amount of either the heme or non-heme Fe tracer recovered in the liver or the spleen.

Table 2.1. Enrichment of ^{58}Fe -heme and ^{57}Fe -non-heme in blood, liver, and spleen in rats¹

| | Non-heme (^{57}Fe) | Heme (^{58}Fe) | <i>P</i> -value |
|------------------------|-------------------------------|---------------------------|-----------------|
| Blood | | | |
| % Fe excess | 8.6 ± 1.6 | 3.8 ± 0.5 | 0.0007 |
| Net tracer amount (μg) | 34.9 ± 6.2 | 1.9 ± 0.3 | 0.0001 |
| % recovered tracer | 80.5 ± 2.4 (n = 11) | 71.3 ± 4.7 (n = 9) | 0.02 |
| Liver | | | |
| % Fe excess | 9.5 ± 3.3 (n = 11) | 3.5 ± 1.3 (n = 10) | 0.002 |
| Net tracer amount (μg) | 6.8 ± 1.1 (n = 11) | 0.41 ± 0.04 (n = 10) | 0.0005 |
| % recovered tracer | 16.8 ± 2.0 (n = 11) | 21.2 ± 3.7 (n = 9) | 0.15 |
| Spleen | | | |
| % Fe excess | 1.6 ± 0.5 | 1.2 ± 0.5 (n = 10) | 0.4 |
| Net tracer amount (μg) | 0.9 ± 0.2 | 0.13 ± 0.03 (n = 10) | 0.002 |
| % recovered tracer | 2.7 ± 0.6 (n = 11) | 7.5 ± 1.8 (n = 9) | 0.01 |

¹ Values are mean ± SEM; n < 12 indicates lack of tissue sample or nickel contamination that interfered with ^{58}Fe determination

Discussion

Few studies have simultaneously assessed the impact of hepcidin on heme and non-heme Fe absorption and tissue utilization in relation to key Fe transport proteins and hepcidin expression. In the rat model of Fe loading, hepcidin was found to have a significantly greater ability to regulate absorption of non-heme Fe when compared to heme Fe, a difference that was accompanied by significant differences in expression of DMT1 and Dcytb. Differential tissue utilization of orally ingested heme versus non-heme Fe was also evident, with the RBC preferentially utilizing Fe derived from a dietary non-heme Fe source while the spleen preferentially utilized dietary Fe ingested in the form of heme Fe.

Moderate Fe loading resulted in a 3-fold increase in liver hepcidin and a 4-fold reduction in non-heme Fe absorption. These changes were accompanied by a marked down-regulation in duodenal *Dmt1* mRNA while duodenal *Fpn* mRNA showed only a slight, but non-significant, decrease in response to Fe injection and increased hepcidin production. Similar findings of an inverse association between hepatic hepcidin expression and duodenal Fe transporter mRNA

have been observed in both Fe-loaded mice (25) and rats (4, 26). These changes are thought to occur in response to rises in intracellular Fe, which could reduce transcript stability via the iron regulatory element/iron regulatory protein system (27), or inhibit transcription by interfering with hypoxia inducible factor-2 signaling (28). We found that transcript levels of *Dmt1* and *Fpn* were highly correlated with their functionally associated ferrireductase/ferroxidase, *Dcytb* and *hephaestin*, suggesting a coordinated regulation of these genes in the duodenum (29, 30). Contrary to the classic hepcidin-dependent down-regulation of FPN protein observed in the macrophage, both FPN protein and transcript expression in the duodenum were unrelated to hepcidin and non-heme Fe absorption. This is in line with recent studies in Caco-2 cells and rodents showing no impact of hepcidin treatment on duodenal FPN and basolateral Fe transport (5, 7, 31). Our results support the accumulating evidence that DMT1, and not FPN, is a primary target for hepcidin regulation in the duodenum. It has been suggested that hepcidin may indirectly affect duodenal FPN protein by down-regulating DMT1 and thus decreasing intracellular Fe concentrations and hence *Fpn* transcripts (5). This may explain the low FPN protein levels in mice with sustained hepcidin expression (5). It is worth noting that hepcidin remained a significant determinant of non-heme Fe absorption after accounting for variation in duodenal *Dmt1* mRNA, suggesting the presence of other absorptive components that mediate the suppression of Fe absorption by hepcidin.

In this study, absorption of Fe from a dietary heme Fe source was on average 60% lower than Fe absorption from non-heme, ferrous sulfate. Our finding of a preferential absorption of non-heme over heme Fe has been previously documented in early radiotracer studies in Sprague Dawley rats where a 70-90% lower retention of Fe from heme Fe sources (Hb (32, 33), myoglobin (34), and beef (35)) was noted compared to Fe retention from non-heme Fe sources.

However, these data are in direct contrast to human studies that consistently show a 2-5 fold greater relative Fe absorption from heme vs. non-heme Fe (36-38). The physiological basis for this species difference in differential heme vs. non-heme Fe utilization is unknown and perhaps reflects adaptation to habitual diets of primarily animal- vs. plant-based Fe sources. Despite the relative differences in heme versus non-heme Fe absorption, cellular mechanisms of heme and non-heme Fe absorption are thought to be similar between these two species (39). There is also ample evidence to suggest that rats respond similarly to dietary and physiological factors known to affect heme Fe absorption in humans including meat proteins and body Fe stores (40). Because of these qualitative similarities, rat models offer important insights on the mechanisms and regulation of heme Fe absorption in humans although species differences must be considered when making inferences.

Both heme and non-heme Fe absorption were suppressed by Fe overload and both were inversely correlated with liver hepcidin. Liver hepcidin expression explained 59% and 63% of the variation in heme and non-heme Fe absorption but had a greater relative impact on non-heme Fe absorption. Liver total Fe concentrations were found to be an equally strong predictor of the absorption of heme and non-heme Fe. These relationships closely resemble those observed between heme absorption and serum ferritin in humans (36, 38), confirming that heme Fe absorption is responsive to changes in body Fe status, though to a lesser extent than non-heme Fe. The inhibitory effect of Fe status on heme Fe absorption did not seem to be mediated by duodenal PCFT and HO-1, as neither was changed by Fe injection or hepcidin. Interestingly, there was a strong inverse relationship between heme absorption and duodenal *Dmt1* mRNA. This may reflect their shared regulation by body Fe status or a role of *Dmt1* in transporting heme-derived inorganic Fe from the endosome after apical endocytosis of heme (9).

The use of stable Fe isotopic techniques provided a unique opportunity to simultaneously compare tissue utilization of these two types of dietary Fe in the same animals in response to body Fe stores. Differential tissue utilization of dietary heme versus non-heme Fe was evident with more non-heme Fe intake being utilized for RBC synthesis while a greater proportion of dietary Fe was delivered to the spleen when ingested as heme Fe. The relative distribution of absorbed non-heme Fe tracer between the liver, spleen, and RBC was similar to prior findings obtained following intravenous injection of radio-Fe salts in mice (41) and rats (42, 43). Few such data are available for heme Fe. While the majority of absorbed heme was recovered in the RBC Fe pool, the fraction of heme tracer recovered in RBC (71.3%) was significantly lower than that observed for non-heme Fe tracer (80.5%). The variable utilization of these two forms of Fe evident in the RBC was supported by the significantly 2-fold higher enrichment of heme-Fe in the spleen when compared to enrichment of non-heme Fe (7.5% vs. 2.7%). There was also a trend for greater uptake of heme Fe in the liver (21.2% vs. 16.8%) but this difference did not reach significance. The differential tissue utilization of absorbed heme versus non-heme Fe may suggest that a portion of absorbed heme exits the enterocyte in a form distinct from non-heme Fe. If heme is exported intact as heme using the FLVCR1 protein found on the basolateral membrane in enterocytes, it should share the same metabolic fate as plasma heme and be taken up by cells that express the heme receptor protein lipoprotein-receptor related protein 1 such as hepatocytes and macrophages in the liver and spleen (44). It has been shown in rats that more than 90% of intravenously injected heme-hemopexin is recovered in liver within 2 hours of tracer injection (45). This is consistent with our finding that a greater proportion of absorbed heme was delivered to the spleen and liver compared to relative tissue utilization of non-heme Fe.

In conclusion, moderate Fe loading in rats significantly increased tissue Fe content and hepatic hepcidin expression without impacting serum ferritin or hemoglobin concentration. Increased hepatic hepcidin expression was associated with significantly lower duodenal absorption of both heme and non-heme Fe absorption. The suppressive effects of hepcidin on non-heme and heme Fe absorption were mediated by DMT1 and were not associated with changes in FPN. Hepcidin more potently down-regulate non-heme absorption than heme Fe absorption in rats. There were significant differences in tissue deposition of the absorbed heme and non-heme Fe tracers suggesting some heme may be exported into the circulation in a different form from non-heme Fe. The cellular and molecular mechanisms underlying this difference will require further study.

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CHAPTER 3

PLACENTAL EXPRESSION OF HEME IRON TRANSPORTERS IN RELATION TO MATERNAL AND NEONATAL IRON STATUS

Study1: Placental heme receptor LRP1 correlates with the heme exporter FLVCR1 and neonatal iron status³

Abstract

LDL receptor-related protein 1 (LRP 1) is a transmembrane receptor highly expressed in the human placenta. It was recently found to be the receptor for heme and its plasma binding protein hemopexin (Hx) and is integral to systemic heme clearance. Little is known about systemic concentrations of hemopexin during pregnancy and whether maternal Hx and placental LRP1 contribute to fetal Fe homeostasis during pregnancy. We hypothesized that placental LRP1 would be up-regulated in maternal/neonatal Fe insufficiency and would be related to maternal circulating Hx. Placental LRP1 expression was assessed in 57 pregnant adolescents (14 -18 y) in relation to maternal and cord blood Fe status indicators (Hb, SF, sTfR), the Fe regulatory hormone hepcidin and serum Hx. Hemopexin at mid-gestation correlated positively with Hb at mid-gestation ($r = 0.35$, $P = 0.02$) and Hx at delivery correlated positively with cord hepcidin ($r = 0.37$, $P = 0.005$). Placental LRP1 protein expression was significantly higher in women who exhibited greater decreases in serum Hx from mid-gestation to term ($r = 0.28$, $P = 0.04$). Significant associations were also found between placental LRP1 protein with cord hepcidin ($r = -0.29$, $P = 0.03$) and placental heme exporter FLVCR1 ($r = 0.34$, $P = 0.03$). Our data are consistent with a role for placental heme Fe utilization in supporting fetal Fe demands.

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Introduction

The placenta serves as the sole conduit for the transfer of maternal nutrients to the fetus across pregnancy. It is believed that most, if not all, of the iron (Fe) delivered to the placenta is supplied as non-heme Fe. It is unclear whether plasma heme obtained from either intravascular red blood cell (RBC) catabolism (1), or from macrophage heme export (2), might also be utilized to support fetal Fe demands.

Hemopexin (Hx) is a plasma protein with high affinity for heme and contributes to systemic Fe homeostasis by delivery heme to the liver for storage (1). Decreases in serum Hx reflect a discharge of heme into the circulation and Hx is commonly measured along with the plasma hemoglobin (Hb) scavenger protein, haptoglobin (Hp), to assess the severity of intravascular hemolysis (3). Few data are available on Hx concentrations in pregnant women and it is unclear whether Hx is related to Fe status.

The LDL receptor-related protein 1 (LRP1) is a trans-membrane protein expressed in a variety of cell types including macrophages, hepatocytes, and neurons (4). Of interest, LRP1 is highly expressed in human placenta (5, 6) and has been suggested to play a role in placental lipid transport (7). Recently, Hvidberg et al. identified LRP1 as the receptor for the Hx-heme complex and revealed a novel role of LRP1 in systemic heme recycling (8). These authors suggested that the high expression of LRP1 in the human placenta may enable Fe recovered from maternal circulating heme to be delivered to the fetus (8) but no studies have examined this hypothesis.

Fe efflux from the placenta to the fetus is thought to be mediated by the only known mammalian cellular non-heme Fe exporter, ferroportin (FPN) (9, 10). Consistent with its role in Fe export, FPN is localized to the basolateral side of human placental syncytiotrophoblast (11). FPN is under tight control by the hormone hepcidin, which binds and induces degradation of FPN protein and thus limits cellular Fe export. Whether this FPN-hepcidin model in

macrophages applies to the human placenta and whether placental FPN is related to expression of heme Fe transporters remain unknown.

The objectives of this study were: 1) to evaluate placental expression of the heme transporter LRP1 and the Fe export protein FPN in pregnant adolescents delivering at term and 2) to investigate the relationships between placental LRP1 and FPN with placental heme Fe exporter FLVCR1, maternal and neonatal Fe status, and changes in maternal Hx across gestation.

Methods

Subjects

Pregnant adolescents in this study were enrolled in a larger prospective study addressing the relationship between maternal and fetal mineral status. All participants were recruited from the Rochester Adolescent Maternity Program in Rochester, NY between 2007 and 2011. Characteristics of this study cohort have been described elsewhere (12, 13) and data on placental expression of Fe and vitamin D-related proteins have been published (14-16). The Institutional Review Boards at Cornell University and the University of Rochester approved all study procedures and written informed consent was obtained from all participants. A maternal blood sample was taken at mid-gestation (~26 wks) and at delivery (~40 wks) at which time cord blood and placental tissue were also obtained. Placental tissue used in this study was from a subset (n = 57) of the 113 teens who delivered term infants.

Biochemical analysis

Blood Hb was measured using a Cell Dyn 4000 hematology analyzer (Abbott Laboratories, Abbott Park, IL). Serum was separated and stored at -80°C prior to analysis. Serum Hx and Hp were determined by sandwich ELISAs from Genway Biotech (Genway

Biotech, San Diego, CA) and ALPCO (ALPCO Diagnostics, Salem, NH), respectively. Serum Hb lower than the normal range (0.3-2.0 g/L) was considered indicative of hemolysis (3). Serum ferritin (SF) and serum soluble transferrin receptor (sTfR) were measured using commercially available ELISA kits (Ramco Laboratories Inc., Stafford, TX). Total body iron (TBI) was calculated from SF and sTfR concentrations using the equation developed by (17): $TBI (mg/kg) = - [\log_{10} (sTfR/SF) - 2.8229] / 0.1207$. Maternal ID was defined as either SF < 12 µg/L, sTfR > 8.5 mg/L, or TBI < 0 mg/kg (18, 19). Because the Hb distribution for African Americans is shifted to the left of that for the Caucasians, we adjusted the Hb cutoffs downward by 0.8 g/dL to define anemia in African American teens as recommended by IOM for black populations (20). Specifically, anemia in whites was defined as Hb < 10.5 g/dL in the second trimester and Hb < 11.0 g/dL in the third trimester and anemia in blacks was defined as Hb < 9.7 g/dL in the second and Hb < 10.2 g/dL in the third trimester. Neonatal anemia was defined when cord blood Hb was < 13.0 g/dL (21). C-reactive protein (CRP) was measured using Immulite 2000 immunoassay system (Seimens Diagnostics, Los Angeles, CA). Intrinsic LifeSciences (La Jolla, CA) measured serum hepcidin using a competitive ELISA specific for the mature peptide. The lower limit for detection of this assay is 5 µg/L. Serum hepcidin concentration below the limit of detection was given a value of 2.5 µg/L for data analysis purposes as previously described for this variable (22).

Placental sample collection

Placentas were collected immediately after delivery. Placental weight and dimensions were recorded. Multiple (4–5) placental samples were collected from different quadrants of the placenta, the maternal and fetal membranes removed and the samples then each cut into quarters and then randomly distributed into aliquots. These aliquots were either flash frozen (for western

blot analyses) or placed into RNAlater (Ambion, Austin, TX) and kept at -80°C. Placental protein lysates were prepared as previously described (14, 15) and protein concentrations of lysates were determined using Bio-Rad dye reagent (Bio-Rad, Hercules, CA). Lysates were diluted in SDS-PAGE sample buffer and stored at -80°C until analysis.

Placental tissue Fe content

Total tissue Fe content of the placental tissue was determined using atomic absorption spectrophotometry as detailed before (14) and expressed as µg Fe/g placental dry weight.

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

The expression of placental *LRPI* mRNA was measured in 42 placentas with quality RNA. Total RNA was extracted from the placental tissue samples using the RNeasy Microarray Tissue Mini Kit (Qiagen, Valencia, CA). The extracted RNA was quantified and verified for integrity by the Experion automated electrophoresis system (Bio-Rad, Hercules, CA). RNA purity was checked by the ratio of absorbance at 260 and 280 nm on a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). A total of 1 µg RNA was reverse-transcribed into cDNA with a transcriptor cDNA synthesis kit (Roche Applied Sciences, Indianapolis, IN). All qRT-PCR reactions were set up in a 10 µL reaction mixture containing 2 µL of the cDNA template, 5 µL of SYBR Green I Master reaction mix, and 0.7 µM of primers and run in triplicate in 384-well plates on a LightCycler 480 instrument (Roche Applied Sciences, Indianapolis, IN). The cycling conditions included an initial denaturation step at 95°C for 5 min, followed by 45 cycles at 95°C for 10 sec, annealing at 60°C for 10 sec, and extension at 72°C for 10 sec. Specificity of amplifications was verified by melt curve analysis after PCR cycles. A single peak was consistently observed in all samples. Relative *LRPI* mRNA expression was normalized to *β-actin* and compared to that obtained from a control placenta sample using

the $2^{-\Delta\Delta Ct}$ method using the following equations: $\Delta Ct = Ct(LRP1) - Ct(\beta\text{-actin})$; $\Delta\Delta Ct = \Delta Ct(\text{sample}) - \Delta Ct(\text{control placenta})$; and $\text{Fold Change} = 2^{-\Delta\Delta Ct}$. The primers were designed using the ProbeFinder software from Roche Applied Science using the NCBI sequence ID (NM_002332 for *LRP1* and NM_001101 for *β -actin*) and purchased from Integrated DNA Technologies (Coralville, IA). Sequences of the primer-pairs were as follows: *LRP1*, Forward: 5' – GAT GAG ACA CAC GCC AATC TG – 3', Reverse: 5' – CGG CAC TGG AAC TCA TCA – 3'; *β -actin*, Forward: 5' – CCA ACC GCG AGA AGA TGA – 3', Reverse: 5' CCA GAG GCG TAC AGG GAT AG – 3'.

Western blot analysis

Western blot was performed to determine placental protein expression of LRP1 and FPN. Placental lysates were separated by SDS-PAGE using a triple-wide electrophoresis unit (CBS Scientific, Del Mar, CA) and electro-transferred to polyvinylidenedifluoride membranes (Millipore, Billerica, MA). Membranes were blocked in Odyssey Blocking Buffer (Li-Cor, Lincoln, NB) for 1 hr and incubated overnight at 4°C with antibodies against the following targets: mouse anti-LRP1 (1:3000 dilution; ab28320, Abcam, Cambridge, MA), rabbit anti-FPN (1:500 dilution; MTP-11A, Alpha Diagnostics, San Antonio), and β -actin (rabbit anti β -actin: 1:5000 dilution: Abcam, Cambridge, MA or mouse anti β -actin: 1:2000 dilution: Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then incubated with appropriate fluorescence-coupled secondary antibodies (Li-Cor, Lincoln, NE) for 1 hr at room temperature. After washing, fluorescence of the protein bands was quantified by with the Odyssey IR imaging system (Li-Cor, Lincoln, NE). A band at 85 kDa corresponding to the LRP1 β -chain was observed in human placenta and rat liver (**Figure 3.1**). A band of a lower molecular weight was evident in the placental lysates and may represent differential phosphorylation of the LRP1 β -chain (23), as

similar patterns have been observed in human placenta (24). The combined fluorescence of the upper and lower bands and that of the upper band was highly correlated ($r = 0.96$) thus the latter was used for data analysis. The use of combined signal of the upper and lower band did not change study findings. A control placenta sample was loaded in the left, middle, and right part of the gel and the CV for the 3 samples was 2.6% indicating minimal influence of the sample location on band intensity. A representative image of FPN western blot is shown in **Figure 3.1**.

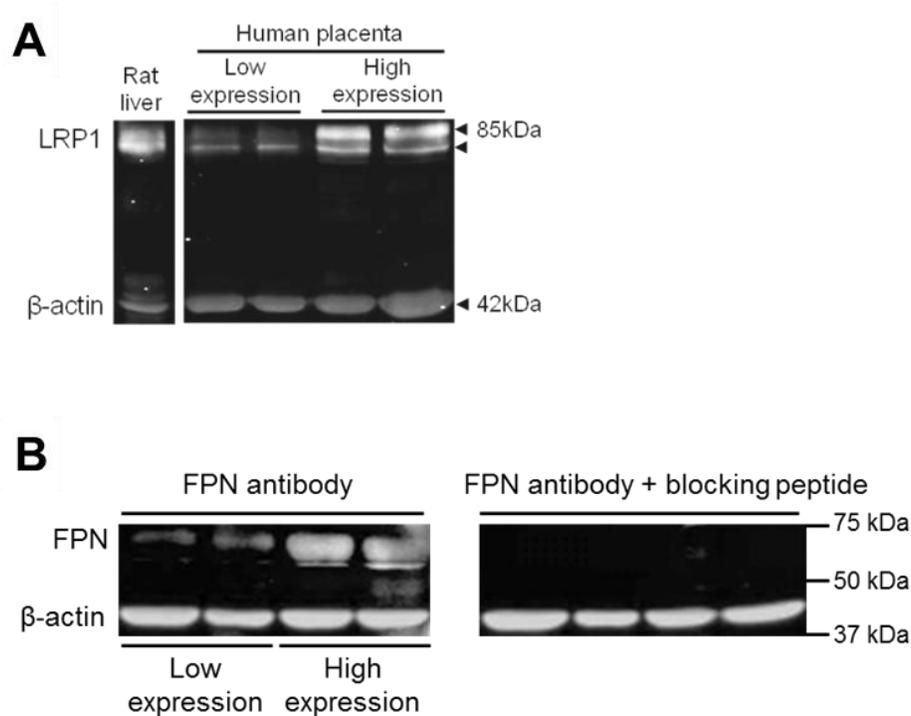


Figure 3.1. (A) Western blot of LRP1 in human placenta. Rat liver was used as a positive control for LRP1. (B) Western blot of FPN in human placenta. The left panel depicts human placental lysates with high and low expression of FPN. Antibody specificity was confirmed by the absence of the FPN band in the blot incubated with a FPN blocking peptide specific to the FPN antibody.

Detailed western blotting methods for the determination of placental FLVCR1 expression in this cohort were previously published (14). Existing data on placental FLVCR1 were used in the present study to explore its relationship with placental LRP1 and FPN.

Statistical analysis

Non-normally distributed variables were transformed to achieve normality prior to data analysis. Paired t-tests were used to compare serum Fe status indicators and Hx between mid-gestation and delivery. Pearson's correlation was used to examine associations between expression of placental LRP1, FPN, and FLVCR1, and serum Fe status indicators. Multivariate analysis was used to study associations while controlling for other variables. Multiple linear regression was used to model placental LRP1 expression. P values < 0.05 were considered significant. Data were reported as the means \pm SDs unless otherwise stated.

Results

Subject characteristics

General characteristics of the study participants are presented in **Table 3.1**. Average age at enrollment was 17.1 y and the majority of adolescents were African American and non-Hispanic. Approximately 19.3% of the adolescents were obese prior to pregnancy and 61.4% gained more than the IOM gestational weight gain recommendations. Of the 57 adolescents, five delivered by Caesarean section. Eleven infants were born small for gestational age and two were large for gestational age.

Maternal Fe status and Hx concentrations across pregnancy

Suboptimal Fe status in this adolescent cohort was evident (**Table 3.2**). Prevalence of anemia was 4.3% at mid-gestation and increased significantly to 10.2% at term. Serum Hx decreased significantly by 9.9% from mid-gestation to delivery ($p = 0.005$). Mid-gestation Hx was positively associated with maternal Hb ($r = 0.35, p = 0.02$) and CRP ($r = 0.30, p = 0.03$) at mid-gestation and with maternal hepcidin ($r = 0.29, p = 0.03$) at delivery. Delivery Hx correlated with maternal hepcidin ($r = 0.28, p = 0.04$) at mid-gestation.

Table 3.1. Characteristics of study participants (n = 57)

| Characteristics | Mean ± SD (Range) |
|--|---------------------------|
| Age at enrollment (y) | 17.1 ± 1.1 (14.0 – 18.7) |
| Parity ≥ 1 (%) | 11 |
| Pre-pregnancy BMI (kg/m ²) | 25.1 ± 6.0 (17.2 – 43.5) |
| Gestational weight gain (kg) | 17.6 ± 7.7 (-2.1 – 43.2) |
| Race (%) | |
| African American | 61 |
| Ethnicity (%) | |
| Non-Hispanic | 75 |
| Delivery mode (%) | |
| Vaginal delivery | 91 |
| Placental weight (kg) | 0.61 ± 0.11 (0.35 – 0.86) |
| Birth weight (kg) | 3.30 ± 0.45 (2.50 – 4.71) |
| Infant gender (%) | |
| Male | 60 |

Table 3.2. Fe status indicators in 57 pregnant adolescents and their neonates¹

| Variable | Mid-Gestation | Delivery | Cord Blood |
|-----------------------|-------------------------------|-------------------------------|--------------------------------|
| Hb (g/dL) | 11.4 ± 0.8 (47) ^a | 11.8 ± 1.3 (49) ^a | 13.7 ± 2.7 (36) ^b |
| SF (µg/L) | 22.2 ± 19.1 (57) ^a | 22.6 ± 12.9 (57) ^a | 133.0 ± 79.4 (57) ^b |
| Serum sTfR (mg/L) | 5.6 ± 5.9 (57) ^a | 5.4 ± 2.7 (57) ^a | 8.3 ± 2.5 (57) ^b |
| TBI (mg/kg) | 3.2 ± 3.8 (57) ^a | 3.6 ± 3.6 (57) ^a | 8.0 ± 2.6 (57) ^b |
| Serum hepcidin (µg/L) | 28.0 ± 19.9 (57) ^a | 36.2 ± 28.4 (57) ^a | 131.8 ± 88.8 (57) ^b |
| Serum Hx (g/L) | 1.0 ± 0.2 (57) ^a | 0.9 ± 0.3 (54) ^b | 0.3 ± 0.2 (47) ^c |
| Serum Hp (g/L) | 0.9 ± 0.5 (57) | ND | ND |
| Serum CRP (mg/L) | 6.6 ± 6.9 (55) ^a | ND | 2.2 ± 10.8 (44) ^b |

¹Data are presented as Mean ± SD (sample size); Sample sizes are different due to missing assay (Hb) or sample availability (serum Hx and CRP); ND, not determined; Values with different subscripts (^{a, b, c}) are significantly statistically different.

Both mid-gestation Hx ($r = 0.50$, $p = 0.002$) and delivery Hx ($r = 0.52$, $p = 0.002$) correlated strongly with cord Hb and remained positive predictors of cord Hb after controlling for maternal Hb at the respective blood sampling point. Higher maternal Hx at delivery was related to better neonatal Fe status as evidenced by its association with high cord hepcidin ($r = 0.37$, $p = 0.005$) and low cord sTfR ($r = -0.27$, $p = 0.046$). There was a non-significant positive correlation between the decrease in Hx from mid-gestation to delivery and the time elapsed

between the two blood sampling points ($r = 0.26$, $p = 0.06$). Greater decreases in Hx across gestation were seen in women with lower mid-gestation hepcidin ($r = -0.27$, $p = 0.046$) and in neonates with lower hepcidin ($r = -0.30$, $p = 0.03$). These relationships remained significant after controlling for time interval between the mid-gestation and delivery measures. Using all the variables obtained, a multivariate model that included weeks elapsed between the biochemical assessments made at mid-gestation and delivery, maternal hepcidin at mid-gestation, and maternal TBI at delivery was found to capture 23.3% of the variation in changes in Hx from mid-gestation to delivery ($p = 0.004$).

Serum Hp was assessed as an additional indicator of hemolysis in maternal mid-gestation blood samples. Serum Hp did not correlate with Fe status indexes in the maternal or neonatal circulation. Four of the 57 teens had low Hp levels (< 0.3 g/L) suggestive of hemolysis but there were no differences in Fe status indicators or hepcidin between these 4 teens and the rest of the cohort. Correlation analysis revealed significant positive associations between serum Hp with serum CRP ($r = 0.31$, $p = 0.02$) and Hx ($r = 0.33$, $p = 0.01$) at mid-gestation.

Neonatal Fe status and Hx concentrations

Neonatal hematological data are presented in **Table 3.2**. Nearly one third of the neonates were anemic at birth (cord Hb < 13 g/dL). Neonatal serum Hx was 74% lower than ($p < 0.0001$), and moderately correlated with maternal Hx at mid-gestation ($r = 0.29$, $p = 0.046$) but not at delivery. Cord Hx was not related to cord Fe status indicators or hepcidin.

Associations between placental LRP1 and FPN with Fe status and Hx concentrations

Expression of LRP1 and FPN in the placenta was not related to maternal race, ethnicity, gestational age at delivery, delivery mode (vaginal delivery, C-section), placental weight, or infant weight. Placental LRP1 protein expression correlated positively with suboptimal neonatal

Fe status as indicated by low cord hepcidin ($r = -0.29, p = 0.03$; **Figure 3.2**) and high cord sTfR ($r = 0.36, p = 0.006$; **Figure 3.2**). Greater decreases in Hx from mid-gestation to delivery were associated with higher placental LRP1 protein expression ($r = 0.28, p = 0.04$; **Figure 3.2**). In the 41 adolescents with data on placental FLVCR1 protein, there was a positive correlation between placental LRP1 and FLVCR1 ($r = 0.34, p = 0.03$; **Figure 3.2**). Placental *LRP1* mRNA was not significantly correlated with LRP1 protein ($p = 0.14$). Greater *LRP1* mRNA tended to be associated with higher placental weight ($r = 0.28, p = 0.08$) and birth weight ($r = 0.26, p = 0.09$). None of the maternal or neonatal Fe status variables were significantly associated with placental LRP1 mRNA.

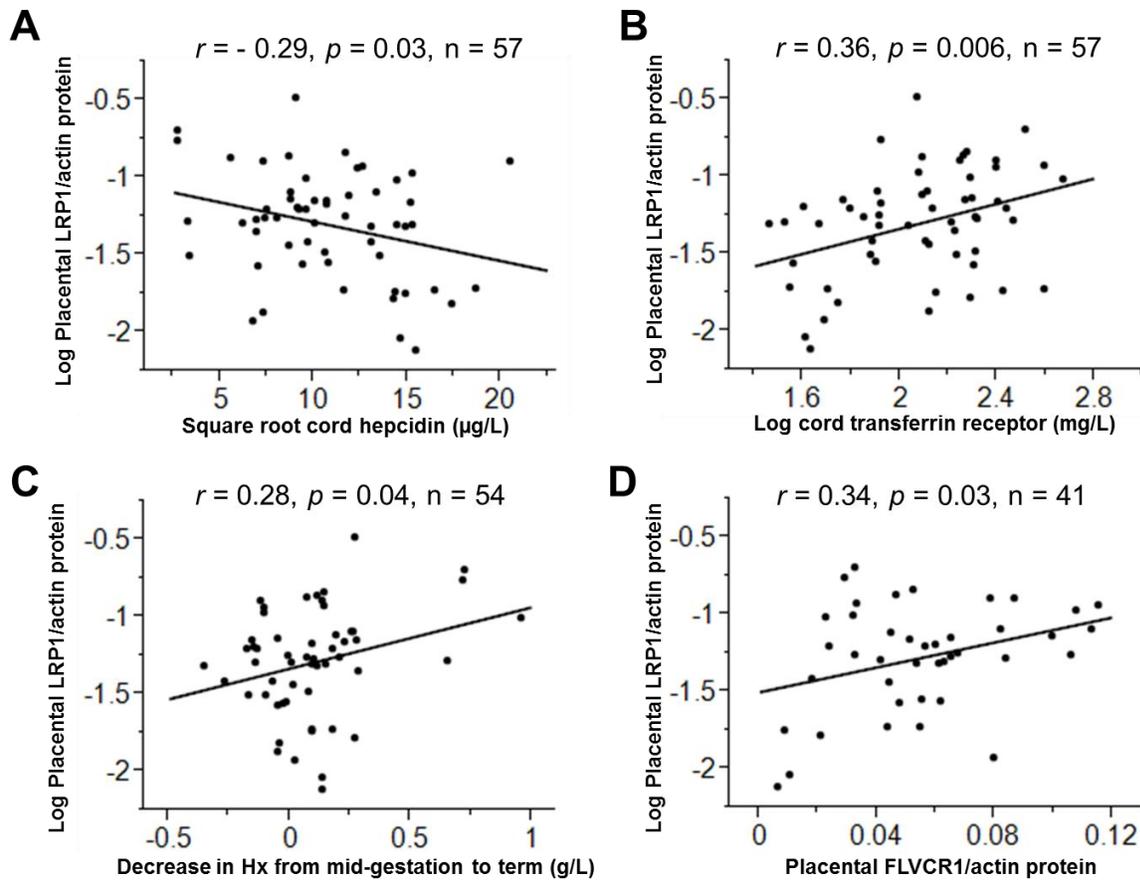


Figure 3.2. Significant correlates of placental LRP1 protein expression in pregnant adolescents

Placental FPN protein expression was insignificantly negatively correlated with maternal serum hepcidin ($r = - 0.23$, $p = 0.08$) and TBI at mid-gestation ($r = - 0.23$, $p = 0.09$). No significant relationship was found between placental FPN with neonatal hepcidin or other Fe status indicators. Similarly, there were no significant relationships between placental FPN and neonatal hepcidin when data were examined in adolescents who were ID or non-ID. Placental Fe content was positively correlated with placental FPN protein expression ($r = 0.37$, $p = 0.01$). Neither LRP1 nor FLVCR1 was correlated with FPN protein expression in the placenta.

Discussion

During late pregnancy, Fe flux across the placenta reaches a maximum of 3-8 mg per day (25), a magnitude several times higher than the amount absorbed from the diet. The high Fe transport capacity of the placenta is partly explained by the abundant presence of TfR on the syncytiotrophoblast (26) and it is believed that transferrin-bound inorganic Fe is the predominant form of Fe utilized by the placenta to support fetal requirements (27, 28). Recently, attention has been focused on the presence of multiple heme transport proteins in the human placenta (29), which suggests that this organ may be an alternate site of heme clearance and/or utilization. In a group of healthy pregnant adolescents, we assessed maternal and neonatal serum Hx in relation to placental expression of the heme-Hx receptor LRP1, the heme exporter FLVCR1, and the non-heme Fe exporter FPN. We documented for the first time a significant positive correlation between two heme transporters LRP1 and FLVCR1 in the human placenta. In addition, placental LRP1 protein expression was higher in neonates with suboptimal Fe status and in women who experienced greater decreases in serum Hx from mid-gestation to delivery. Finally, we report

novel associations between maternal Hx with neonatal Hb and hepcidin, adding to the biological plausibility of a role for plasma heme in maintaining Fe homeostasis during pregnancy.

Circulating Hx and liver LRP1 facilitate plasma heme clearance and protect against heme-associated oxidative damage. The importance of Hx in the resolution of hemolysis is evident from knockout studies showing that mice lacking Hx are more sensitive to both induced hemolysis (30) and heme overload (31). In addition to systemic heme scavenging, Hx and LRP1 may mediate localized heme clearance in the brain during cerebral hemorrhage (32). Functional analyses have demonstrated the Hx-heme binding activity of LRP1 purified from human placenta (8), but few studies have examined the potential role of placenta as a site of Hx-heme clearance. In our study, placental protein expression of LRP1 was positively associated with that of FLVCR1, a plasma membrane heme exporter essential for erythroid cell survival and macrophage heme Fe recycling (33). It is possible that placental heme uptake (mediated by LRP1) and heme export (mediated by FLVCR1) are coordinately regulated to allow for sequestration of circulating heme while preventing accumulation of intracellular heme as depicted in **Figure 3.3**.

Little is known about the cellular regulation of LRP1 expression by systemic Fe status. Studies in mouse hepatoma cells have found that Fe deprivation doubled the number of LRP1 and increased heme uptake, indicating that LRP1 may be negatively regulated by Fe availability (34). Consistent with this reciprocal Fe regulation of LRP1 in the liver, we found that high placental LRP1 was associated with suboptimal neonatal Fe status, which may represent a compensatory mechanism to increase placental heme uptake during Fe insufficiency in support of fetal demands.

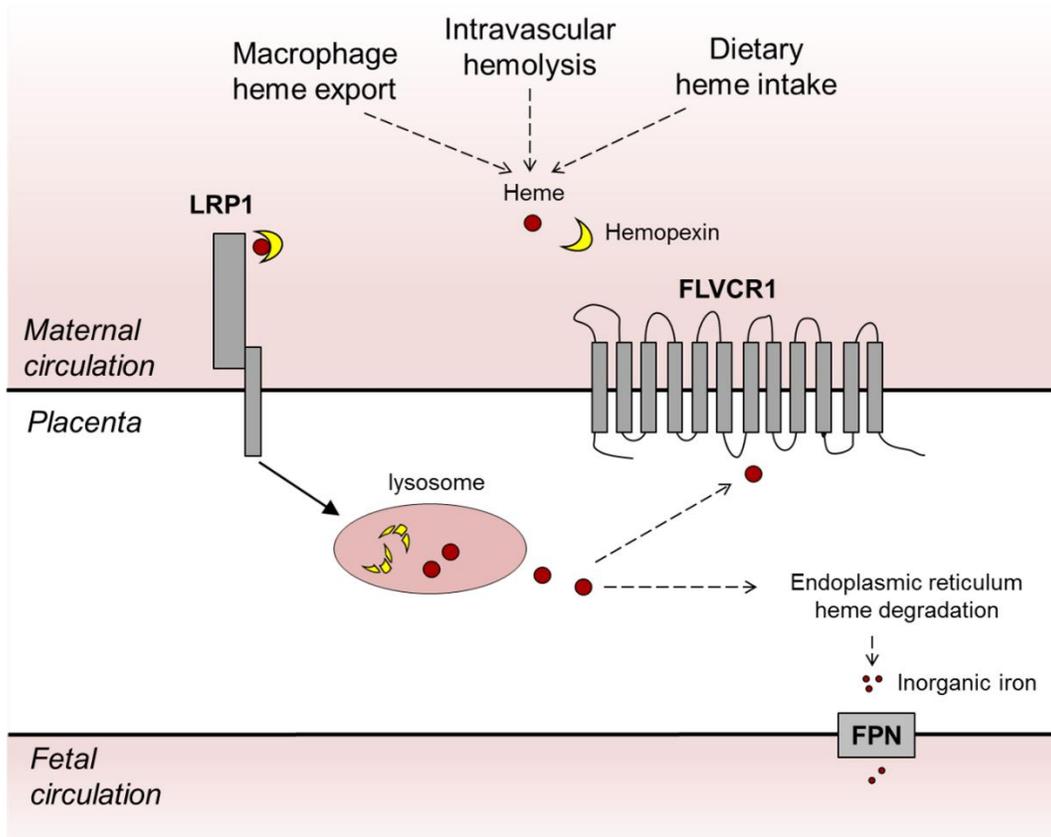


Figure 3.3. Proposed pathway of heme Fe utilization in the placenta

Knockout experiments and localization studies provide convincing evidence that FPN is the protein that mediates Fe exit from placenta to the fetal circulation (10, 11, 35). At this time it is unclear whether levels of FPN protein in the placenta are controlled by hepcidin in a similar way to that found in macrophages. Our study, and previous research in rodents (36-38) and humans (39), suggests that placental FPN protein is not regulated by maternal or neonatal Fe status. These data support the notion that FPN-mediated Fe export may not be the rate-limiting step for placental Fe transfer (37). However, it still may be possible that changes in FPN subcellular location may inhibit Fe export without increased proteolytic degradation.

The Hx concentrations observed in maternal and neonatal circulation were within the ranges previously established for healthy adults (3) and term infants (40). Similar to previous

reports (41, 42), cord Hx concentrations were 74% lower than those observed in maternal circulation, a phenomenon that has been suggested to be a consequence of low fetal Hx production (43).

Kinetic studies demonstrated that extracellular Hx enhances heme export from human macrophages, supporting an emerging role of Hx in systemic heme trafficking (2). It has been suggested that the high serum concentration of Hx, which is comparable to that of the non-heme Fe transporter transferrin, is supportive of the importance of Hx in macrophage heme recycling under physiological conditions (33). Consistent with a role of Hx in systemic Fe balance in pregnancy, we observed associations between maternal Hx with cord Hb and hepcidin. Few studies have examined possible relationships between Hx and Fe status. In diabetic patients and healthy volunteers (n = 213), serum transferrin was a weak predictor of serum Hx while serum tri-acylglycerol and the diabetic state accounted for the majority of the variation (44). It is possible that the positive association between maternal Hx with maternal and cord Hb is representative of a greater capacity of maternal heme recycling and transport in support of fetal erythropoiesis.

The major strength of this study is the concurrent measurement of placental expression of LRP1 and FLVCR1 protein expression with serum Hx in a group of pregnant adolescents with well-characterized Fe status. Due to the observational nature of this study, we could not determine cellular processes underlying the associations between LRP1, Hx, and Fe status and mechanistic studies are needed to assess the heme transport activity of placental LRP1 and determine whether this process is responsive to changes in maternal/neonatal Fe status.

In summary, this study extends the current discussion of heme utilization to the placenta by documenting a relationship between placental LRP1 with maternal and neonatal Fe status in

pregnant adolescents with term delivery. Because of the observational nature of this study, further research is needed to elucidate the mechanisms underlying these associations and examine the significance of placental heme scavenging in supporting fetal Fe demands.

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CHAPTER 3

PLACENTAL EXPRESSION OF HEME IRON TRANSPORTERS IN RELATION TO MATERNAL AND NEONATAL IRON STATUS

Study 2: Maternal obesity has no impact on placental folate transporter expression and cord folate concentrations in pregnant adolescents⁴

Abstract

Negative effects of maternal obesity during pregnancy may be mediated in part by the impact of increased circulating leptin on placental folate transport. The determinants of folate transporter expression in the placenta are not well defined. The role of the cellular folate and heme transporter, proton-coupled folate transporter (PCFT), in the placenta unknown. We assessed placental expression of three major folate transporters (proton coupled folate transporter (PCFT), reduced folate carrier (RFC), and folate receptor α (FR α)) in relation to maternal obesity, circulating leptin and Fe and folate status indicators in 49 pregnant adults and adolescents with term deliveries. Folate and Fe status indicators (Hb, serum folate, hepcidin, SF, and erythropoietin) and serum leptin were assessed in late pregnancy (28.8 ± 5.0 wks) and at delivery in the mother and the neonate. Placental folate transporter protein (PCFT and RFC) and transcript (PCFT, RFC and FR α) expression was assessed. Nearly one third of the participants were obese prior to pregnancy but maternal ppBMI or GWG did not impact placental folate transporter expression. A trend for higher cord folate in neonates born to obese mothers approached significance ($p=0.06$). Circulating leptin in late pregnancy was positively associated placental mRNA expression of RFC and FR α . Unlike RFC and FR α , placental PCFT was not

⁴ Cao C, Pressman EK, Cooper EM, O'Brien KO. Submitted to *Journal of Nutrition* July 2014.

correlated with cord folate concentration but was positively associated with serum hepcidin and erythropoietin in the neonate. Maternal obesity and increased leptin concentrations did not significantly impact cord serum folate concentrations or placental folate transporter expression in healthy pregnant adolescents at term. The association between PCFT and cord Fe status suggests a possible role of PCFT in placental Fe transport.

Introduction

During pregnancy, folate demands increase by 5- to 10-fold to ensure adequate supply for DNA synthesis, cell proliferation, placental development and fetal growth (1). Maternal folate deficiency is associated with various negative pregnancy and neonatal outcomes including preterm delivery, low birth weight, congenital abnormalities, and neural tube defects (NTDs) (1-3).

The placenta transports folate from the maternal circulation to the fetus against a concentration gradient (4). This process is thought to be facilitated by the polarized distribution of folate transporters in the syncytiotrophoblast (5). Folate receptor alpha (FR α), a glycosylphosphoinositol-linked protein highly polarized to the maternal side of the human placenta (6, 7), mediates the uptake of folate from the maternal circulation while reduced folate carrier (RFC) is responsible for basolateral folate export into the fetal circulation (6, 8). This classic model of placental folate transport has been updated to incorporate the recently identified cellular folate transporter, proton-coupled folate transporter (PCFT) (9). Loss-of-function mutations in PCFT result in systemic folate deficiency (9, 10) and are the molecular basis for hereditary folate malabsorption in humans. In addition to folate, PCFT also exhibits some affinity for heme Fe and has been suggested to play a role in intestinal heme absorption (11). In human intestinal Caco-2 cells, transfection of PCFT enhances heme uptake (11, 12) while inhibition of PCFT reduces heme uptake (12, 13). The human placenta abundantly expresses PCFT (9) but the role of PCFT in this organ is much less understood. In the human syncytiotrophoblast, PCFT co-localizes with FR α on the maternal-facing membrane (6, 7), suggesting an involvement of PCFT in placental folate uptake. However, no PCFT-mediated folate transport activity could be demonstrated in human placental villous membranes when

studied under physiological pH conditions (14), which is consistent with the low pH optimum required for PCFT function (9). In addition, unlike *Fra*^{-/-} and *Rfc*^{-/-} mice that die early *in utero* (15, 16), *Pcft*^{-/-} animals survive through embryonic development (10), which indicates a redundant role of PCFT in placental folate transport.

It has been suggested that maternal obesity may negatively affect placental folate transport (14) and contribute to the higher incidence of neural tube defects observed among obese pregnant women (17). This effect may be mediated by obesity associated hyperleptinemia, as supported by experiments in human trophoblast BeWo cells (18). Human studies are needed to assess possible associations between placental folate transporters and maternal and neonatal folate status and to examine the impact of maternal pre-pregnancy BMI (ppBMI) and gestational weight gain (GWG), and adipokines on placental folate transport and cord folate and Fe concentrations.

The purpose of this study was to assess placental expression of all known folate transporters (PCFT, RFC, and FR α) in relation to maternal and neonatal folate and Fe status, and to explore the possible impact of maternal obesity and circulating leptin on placental folate transporter expression and cord serum folate concentrations in a group of healthy pregnant participants delivering term infants.

Methods

Subjects

Placental tissue (n=49) utilized in this study was obtained from two studies examining relationships between maternal and neonatal nutritional status and placental transporter expression during pregnancy. Pregnant adults (≥ 20 y) and adolescents (13-19 y) were recruited

from the University of Rochester Medical Center Midwifery Group and the Rochester Adolescent Maternity Program and in Rochester NY. Females were eligible to participate if they were otherwise healthy and carrying a single fetus. Exclusion criteria included HIV infection, diabetes, and malabsorption diseases. Both studies were approved by the Institutional Review Boards at Cornell University and the University of Rochester and informed consent was obtained from all participants. Data on maternal and fetal bone growth and calcitropic hormones (19, 20), placental expression of vitamin D metabolic enzymes (21) and Fe transporters (22-24), and maternal Fe absorption and placental Fe transfer (25, 26) have been previously published.

The study design and sample collection of the two parent studies are detailed elsewhere (20, 22). In brief, information on maternal race, ethnicity, height, pre-pregnancy weight, and cigarette smoking was self-reported upon study entry. Weight at delivery was recorded by clinical staff using standard procedures and gestational weight gain was calculated by subtracting pre-pregnancy weight from delivery weight. Maternal blood samples (10 mL) were obtained during pregnancy (28.8 ± 4.9 wks) and at delivery (39.9 ± 1.0 wks). Cord blood samples were also obtained at delivery. Placentas were processed immediately after delivery and placental weight and dimensions were recorded. Multiple (4–5) tissue samples were collected from different quadrants of the placenta, the maternal and fetal membranes removed and the samples each cut into quarters and randomly distributed into aliquots. These aliquots were either flash frozen (for western blot analyses) or placed into RNAlater (Ambion, Austin, TX) and kept at -80°C until analysis. Placental tissues used in this paper were obtained from a subset ($n = 49$) of the 97 participants with both term deliveries (> 37 wks of gestation) and quality placental RNA and protein available.

Biochemical analysis

Hemoglobin (Hb) was measured by the clinical laboratory at the Strong Memorial Hospital (Cell Dyn 4000 hematology analyzer; Abbott Laboratories, Abbott Park, IL). Serum ferritin (SF) and serum soluble transferrin receptor (sTfR) were measured using ELISA kits (Ramco Laboratories Inc., Stafford, TX). Total body iron (TBI) was calculated using the equation developed by Cook et al: $TBI \text{ (mg/kg)} = - [\log_{10} (\text{serum sTfR/SF}) - 2.8229] / 0.1207$ (27). Maternal ID was defined as either $SF < 12 \mu\text{g/L}$, $sTfR > 8.5 \text{ mg/L}$, or $TBI < 0 \text{ mg/kg}$ (28, 29). Anemia in Caucasian participants was defined as Hb values $< 10.5 \text{ g/dL}$ in the second trimester or $< 11.0 \text{ g/dL}$ in third trimester (30). For African American participants, the Hb cutoffs for anemia were adjusted down by 0.8 g/dL based on IOM recommendations (31). Neonatal anemia was identified when cord blood Hb was $< 13.0 \text{ g/dL}$ (32).

Serum folate, vitamin B₁₂ and erythropoietin (EPO) were measured using the Immulite 2000 immunoassay system (Seimens Medical Solutions Diagnostics, Los Angeles, CA). Due to limited sample volume in maternal delivery and cord blood samples, serum folate and B₁₂ were only assessed in 31 of the 49 women and 39 of the 49 neonates at delivery. Folate deficiency was defined as a serum folate concentration $< 6.8 \text{ nmol/L}$ and vitamin B₁₂ deficiency was identified as a serum concentration $< 148 \text{ pmol/L}$ (33). Serum leptin concentrations were determined by ELISA (Millipore, Billerica, MA). Intrinsic LifeSciences (La Jolla, CA) and serum hepcidin was measured using a competitive ELISA specific for the mature hepcidin peptide (34). The lower limit for detection of this assay is $5 \mu\text{g/L}$. Hepcidin concentrations below the limit of detection were given a value of $2.5 \mu\text{g/L}$ for data analysis purposes as previously reported (23).

Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the placental tissue using the RNeasy Microarray Tissue Mini Kit (Qiagen, Valencia, CA). Extracted RNA was quantified and verified for integrity by the Experion automated electrophoresis system (Bio-Rad, Hercules, CA). RNA purity was checked by the absorbance ratios at 260 and 280 nm on a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). A total of 1000 ng RNA was reverse-transcribed using a transcriptor cDNA synthesis kit (Roche Applied Sciences, Indianapolis, IN). The reaction mix was incubated for 10 min at 25°C, followed by 30 min at 55°C and 5 min at 85°C. The cDNA sample were then stored at -20°C until qPCR analysis. The qPCR primers were designed through Roche Applied Science Universal Probe Library using the NCBI sequence ID (NM_016725 for *Fra*, NM_194255 for *Rfc*, NM_080669 for *Pcft*, NM_001101 for β -*actin*) and purchased from Integrated DNA Technologies (Coralville, IA). The primer sequences were as follows: *Ffa*, F: 5' - CTGAGCCAGACGGAGAGC - 3', R: 5' - CTGTGTTGTCATCCGCTGAG - 3'; *Rfc*, F: 5' - CAGTTCCTCGTGCCCATC - 3', R: 5' - GGCAAAGAACGTGTTGACC - 3'; *Pcft*, F: 5' - CATCCCGGCTGTTCTGAT - 3', R: 5' - CTGCTGGAACTCGAGGTGA - 3'; β -*actin*, F: 5' - CCAACCGCGAGAAGATGA - 3', R: 5' - CCAGAGGCGTACAGGGATAG - 3'. The qPCR reactions were prepared in a 10 μ L reaction mixture (2 μ L of cDNA template, 5 μ L SYBR Green I Master reaction mix, 0.35 μ L of 20 μ mol/L primers, and 2.3 μ L of H₂O) and run in triplicate in 384-well plates on a LightCycler 480 instrument (Roche Applied Sciences, Indianapolis, IN). The cycling conditions included a denaturation step at 95°C for 5 min, 45 cycles of 95°C each for 10 sec, annealing at 60°C for 10 sec, and extension at 72°C for 10 sec. A no-reverse transcriptase control and a no-template control were used in all qPCR experiments to detect DNA contamination in RNA samples and PCR reagents. To ensure the purity of the

amplification product, a melt curve was generated for each PCR reaction by holding the temperature at 95°C for 5 sec then 65°C for 1 min followed by a gradual increase in temperature to 97°C at a rate of 0.11°C/sec. Melt curves were consistent with a single gene product in each reaction. Relative mRNA expression of *Fra*, *Rfc* and *Pcft* was normalized to β -*actin* and compared to that obtained from a control placental sample using the $2^{-\Delta\Delta Ct}$ method and the following equation: $\Delta Ct = Ct (Fra, Rfc \text{ or } Pcft) - Ct (\beta\text{-actin})$; $\Delta\Delta Ct = \Delta Ct (\text{sample}) - \Delta Ct (\text{control placenta})$; and $\text{Fold Change} = 2^{-\Delta\Delta Ct}$.

Western blot analyses

Protein lysates for western blot experiments were prepared by homogenizing placental tissue in hypertonic lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) as previously described (25, 26). Protein concentrations of the lysates were determined by the Bradford dye-binding method (Bio-Rad, Hercules, CA). Placental lysates were diluted in SDS sample buffer and stored at -80°C until analysis

Due to the lack of suitable commercially available FR α antibody, western blot was only performed for PCFT and RFC. Protein samples were separated by 10% SDS-PAGE, electro-transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA), and blocked in Odyssey Blocking Buffer (Li-Cor, Lincoln, NB). The blots were then incubated overnight at 4°C with rabbit anti-PCFT (1:1000; ab25134, Abcam, Cambridge, MA) or rabbit anti-RFC (1:1000; sc-98970, Santa Cruz Biotechnology Inc., Santa Cruz, CA). β -actin was used as the loading control (1:5000 dilution; sc-47778; Santa Cruz Biotechnology Inc.) and was probed simultaneously with the folate transporter protein targets. Protein targets were detected with IR800 goat anti-rabbit IgG (1:5000; Li-Cor, Lincoln, NE) and quantified by the Odyssey infrared imaging system (Li-

Cor). Human small intestine and rat kidney were used as the positive controls for PCFT and RFC, respectively.

Dietary nutrient intake

Frequency of prenatal supplement use was self-reported at study enrollment. In the 37 subjects participating in the bone health study, information on dietary folate and Fe intake was also obtained by 24-h dietary recall and analyzed by a registered dietitian using the Nutrition Data System for Research (University of Minnesota, Minneapolis, MN).

Statistical analysis

All statistical analyses were performed using JMP Pro 10 (SAS Institute, Cary, NC). Non-normally distributed variables were transformed before further analysis. Two tailed t-tests were performed to compare placental transporter expression between ppBMI and GWG categories. Pearson's correlation was used to assess the relationships between placental folate transporters with ppBMI, GWG, serum leptin, and folate and Fe status indicators. Forward stepwise regression was utilized to identify significant determinants of placental expression of folate transporters and cord folate concentrations. Significance was set at 0.05 and p values < 0.1 were considered as trending toward significance. Data are reported as the means \pm SDs unless specified otherwise.

Results

General characteristics of the study participants are shown in **Table 3.3**. This study included 6 pregnant adults (21.7 ± 2.7 yrs) and 43 pregnant adolescents (17.2 ± 1.2 yrs). There were no significant differences between the adult and adolescent participants in ppBMI, GWG, leptin, and maternal and neonatal Fe status indicators (Hb, SF, sTfR, TBI, EPO, and hepcidin).

More than half of the participants (55%) entered pregnancy overweight or obese while 69% experienced excessive weight gain across gestation based on ppBMI. Fully 55% of participants were taking prenatal supplements every day during pregnancy while 41% reported a frequency of 1-5 times/week. Women who reported daily supplement use tended to have higher serum folate concentrations in late gestation than those who consumed supplements with lower frequency (34.7 vs. 44.5 nmol/L, $p=0.1$). Of the 37 participants with data on dietary nutrient intake, 27% and 59% did not meet the estimated average requirement (EAR) for folate (520 $\mu\text{g/day}$) (38) and Fe (23 mg/day) (39), respectively. However, there was no significant difference in maternal and cord serum folate concentrations between the participants whose folate intake was below EAR compared with those with intakes above the EAR.

Table 3.3. Characteristics of pregnant subjects (n=49)

| Characteristics | Mean \pm SD |
|--|-----------------|
| Age at enrollment (y) | 17.7 \pm 2.1 |
| Gestational age at delivery (wk) | 39.9 \pm 1.0 |
| Parity \geq 1 (%) | 20 |
| Pre-pregnancy BMI (kg/m^2) | 26.6 \pm 5.9 |
| Gestational weight gain (kg) | 17.2 \pm 7.4 |
| Cigarette use during pregnancy (%) | 10 |
| Daily prenatal supplement use (%) | 55 |
| Dietary folate intake ($\mu\text{g/day}$) ^a | 840 \pm 392 |
| Dietary Fe intake (mg/day) ^a | 20.1 \pm 7.6 |
| Race (%) | |
| African American | 61 |
| Caucasian | 39 |
| Ethnicity (%) | |
| Hispanic | 33 |
| Non-Hispanic | 67 |
| Placenta weight (kg) | 0.61 \pm 0.10 |
| Infant weight (kg) | 3.31 \pm 0.41 |

^a Available for 37 of the participants in the maternal and fetal bone health study

Maternal and neonatal folate status and maternal obesity

This study cohort exhibited low Fe status but normal folate status (**Table 3.4**). Anemia was evident in 10.4% of the participants at delivery and 20.4% of the participants had depleted Fe stores (TBI < 0 mg/kg). All participants had normal B12 concentrations in late gestation while 19% (6/31) were B12 deficient at delivery. None of the participants had folate deficiency in late gestation or at delivery. Cord serum folate was 1.6-times higher than ($p < 0.0001$, $n = 26$), and strongly correlated with maternal serum folate at delivery ($r = 0.66$, $p = 0.003$).

Table 3.4. Fe and folate status in 49 pregnant participants and their neonates¹

| Variable | Late gestation (28.8 ± 4.9 wks) | Delivery (39.9 ± 1.0 wks) | Cord Blood |
|----------------------------|------------------------------------|------------------------------|----------------------------|
| Hb (g/dL) | 11.2 ± 0.8 ^a | 11.5 ± 1.3 ^a | 14.2 ± 2.9 ^b |
| SF (µg/L) | 21.8 ± 20.3 ^a | 25.5 ± 17.0 ^a | 144.7 ± 97.9 ^b |
| Serum sTfR (mg/L) | 5.5 ± 5.3 ^a | 6.0 ± 3.2 ^a | 8.1 ± 2.5 ^b |
| TBI (mg/kg) | 2.7 ± 4.4 ^a | 3.3 ± 4.0 ^a | 8.3 ± 2.6 ^b |
| Serum EPO (mIU/mL) | 36.8 ± 21.2 | 36.4 ± 24.1 | 40.7 ± 35.5 |
| Serum hepcidin (µg/L) | 22.1 ± 19.3 ^a | 34.1 ± 36.5 ^a | 141.8 ± 101.7 ^b |
| Serum folate (nmol/L) | 39.9 ± 20.0 ^a | 43.0 ± 16.9 ^a | 70.2 ± 26.0 ^b |
| Serum vitamin B12 (pmol/L) | 319.0 ± 123.7 ^a | 257.3 ± 114.1 ^b | 746.5 ± 454.3 ^c |
| Serum leptin (ng/mL) | 33.1 ± 20.5 ^a | 42.7 ± 22.0 ^a | 11.6 ± 9.3 ^b |

¹Data are presented as Mean ± SD. Values with different subscripts (^a, ^b, ^c) are significantly different

Women who were obese prior to pregnancy had significantly higher serum folate than the non-obese participants at delivery (53.8 ± 19.2 vs. 39.3 ± 14.7 nmol/L, $p = 0.04$). This difference remained significant after adjusting for dietary folate intake. In addition, neonates born to obese mothers tended to have higher serum folate than those born to non-obese mothers ($p = 0.06$). Maternal serum leptin in late gestation was significantly higher in obese mothers and tended to be positively associated with cord serum folate ($r = 0.31$, $p = 0.07$).

Determinants of placental folate transporter expression

None of the placental folate transporters (transcript or protein) were related to maternal age, ethnicity, race, parity, infant weight, frequency of supplement use, or adequacy of dietary folate and Fe intake. The five participants who self-reported smoking during pregnancy had significantly higher placental RFC protein expression compared with the non-smokers ($p=0.02$). Placental RFC mRNA levels tended to decrease with advancing gestational age ($p=0.1$). There was a trend for a positive association between placental PCFT mRNA and placental weight ($p=0.08$). Male infants tended to have higher placental FR α mRNA expression than female infants ($p=0.06$).

We examined the interrelationships between the expression of the three folate transporters in the placenta and their individual relationships with maternal and cord folate concentrations. Placental FR α mRNA expression was positively correlated with both RFC mRNA ($r=0.52$, $p=0.0001$) and PCFT mRNA ($r=0.39$, $p=0.007$). There was no relationship between placental RFC and PCFT at the mRNA or protein level. Cord serum folate concentrations were positively associated with placental mRNA expression of FR α ($r=0.32$, $p=0.048$) and RFC ($r=0.32$, $p=0.045$), but not with PCFT mRNA ($p=0.75$) or PCFT protein ($p=0.83$). Placental RFC protein expression tended to be inversely associated with maternal serum folate in late gestation ($r=-0.28$, $p=0.06$).

Associations between serum leptin and placental folate transporters

We explored the relationships between placental folate transporter expression and obesity-related variables including ppBMI, GWG, and maternal and cord serum leptin. Pre-pregnancy BMI did not impact placental mRNA or protein expression of the three folate transporters. Placental RFC mRNA was positively correlated with GWG ($r=0.35$, $p=0.01$) and

was 1.7-times higher in women who experienced excessive GWG compared with those who gained within or under the recommended range ($p=0.002$). Serum leptin in late gestation was positively correlated with placental mRNA expression of $FR\alpha$ ($r=0.52$, $p=0.0002$) and RFC ($r=0.42$, $p=0.003$). Maternal leptin in late pregnancy remained a significant predictor of placental $FR\alpha$ mRNA ($p=0.0001$) and RFC mRNA ($p=0.003$) after controlling for the gestational age at which the blood was collected. There was a non-significant trend for a correlation between cord leptin and placental RFC mRNA ($r=0.25$, $p=0.07$). Neither placental PCFT mRNA nor protein was significantly associated with maternal or cord leptin.

Because PCFT may also function as a cellular heme transporter and folate and Fe are both essential for erythropoiesis, we examined possible relationships between placental expression of PCFT, RFC, and $FR\alpha$ with maternal and cord Fe status. Placental PCFT mRNA was negatively associated with cord EPO ($r=0.32$, $p=0.03$) and insignificantly correlated with cord SF ($r=0.28$, $p=0.053$). A significant positive correlation was found between placental PCFT protein expression and cord hepcidin ($r=0.28$, $p=0.05$). Placental $FR\alpha$ mRNA was positively correlated with maternal Hb in late pregnancy ($r=0.37$, $p=0.01$) and at delivery ($r=0.32$, $p=0.03$). RFC expression in the placenta was not related to maternal or neonatal Fe status indicators examined.

Multiple regression models were constructed for each placental folate transporters and cord folate concentrations (**Table 3.5**). Maternal leptin in late gestation was a significant predictor of both RFC and $FR\alpha$ in the placenta while cord Fe status variables best captured the variations in placental PCFT mRNA and protein expression. A model that included maternal serum folate at delivery and placental $FR\alpha$ mRNA expression explained 54% of the variability in cord serum folate ($p=0.0002$).

Table 3.5. Multivariate models of placental folate transporters and cord serum folate

| | R ² (Model) | β | P-value |
|--|------------------------|---------|---------|
| Placental RFC | | | |
| RFC mRNA (n = 47) | 0.24 | | 0.003 |
| Log maternal leptin in late gestation | | 0.17 | 0.03 |
| Excessive gestational weight gain | | 0.07 | 0.08 |
| RFC protein (n=47) | 0.15 | | 0.03 |
| Log maternal folate in late gestation | | -0.20 | 0.05 |
| Cigarette smoking | | 0.14 | 0.05 |
| Placental PCFT | | | |
| PCFT mRNA (n=47) | 0.18 | | 0.01 |
| Log cord EPO | | -0.09 | 0.03 |
| Log cord SF | | 0.08 | 0.07 |
| PCFT protein (n=49) | 0.12 | | 0.02 |
| Sqrt cord hepcidin | | 0.02 | 0.02 |
| Placental FRα mRNA (n=47) | | | |
| Log maternal leptin in late gestation | 0.33 | 0.29 | 0.0001 |
| Male infant | | 0.08 | 0.07 |
| Cord serum folate (n=26) | | | |
| Log maternal serum folate at delivery | 0.54 | 0.53 | 0.0005 |
| Sqrt placental FR α mRNA | | 0.36 | 0.03 |

Discussion

In a group of pregnant subjects with a high prevalence of obesity (27%) and excessive GWG (69%), there was no evidence for a detrimental effect of obesity or excessive GWG on maternal and neonatal folate status or on placental folate transporter expression. We observed strong positive associations between maternal leptin and expression of RFC and FR α in the placenta and documented for the first time positive correlations between placental transcript expression of *Rfc* and *Fra* with cord serum folate, which is consistent with a major role of RFC and FR α in placental folate transport. In contrast, placental protein/transcript expression of PCFT was not related to cord serum folate but was significantly associated with neonatal Fe status, which is consistent with a link between PCFT and placental Fe utilization.

None of the study participants were folate deficient during pregnancy and the median folate concentration of this group was comparable to both US women of child-bearing age (33) and to concentrations observed among pregnant women in Australia where folate fortification is also mandatory (37). Consistent with the active nature of placental folate transport, cord folate concentrations were 1.8-times higher than, and significantly correlated with, maternal serum folate at delivery.

It has been suggested that maternal obesity may limit fetal folate availability and thus contribute to increased risk of developmental defects associated with maternal obesity (38). We found that neonates born to obese mothers had comparable folate concentrations compared with those born to non-obese mothers. This finding is consistent with results presented in two small case-control studies (n=30 in each study) that assessed associations between maternal obesity and cord folate status (14, 39). Consistent with previous research (14, 39), we also found no evidence for inadequate folate supply as a function of maternal obesity as neither dietary folate intake nor serum folate levels during pregnancy differed between the obese and non-obese groups. However, as suggested by Carter et al. (14), the lack of an association between maternal obesity and cord folate at term does not preclude the possibility that maternal obesity may limit fetal folate availability early in gestation resulting in developmental defects. Animal models are needed to investigate the impact of maternal obesity on fetal folate availability at earlier stages of gestation and to examine other potential mechanisms underlying the link between maternal obesity and risk of adverse birth outcomes.

The positive associations between cord serum folate and placental *Fra* and *Rfc* are consistent with the major role that these two proteins are thought to play in delivering folate to

the fetus. In addition, placental transcript levels of *Fra* and *Rfc* were highly correlated, which may suggest a shared regulatory pathway to facilitate placental folate trafficking.

Because leptin has been shown to suppress folate transport in human trophoblast BeWo cells (18), we hypothesized that placental expression of folate transporters would be negatively associated with maternal leptin concentrations. In contrast, a positive association between maternal serum leptin and placental FR α and RFC expression was observed, which may reflect the role of leptin as an integrating signal that modulates placental nutrient transport as a function of maternal nutrient availability (40). The discrepancy between our findings and earlier cell culture data may be due to the non-physiological, 100-times higher concentration of leptin used to elicit alterations in folate transport in the BeWo cell model. Data from the BeWo cell culture study also suggested that leptin may impact the Janus-Kinase 2 signaling transduction pathway (18). Additional studies are needed to determine the effect of leptin on placental folate transport at doses that resemble the physiological concentrations observed during pregnancy.

Although the role of PCFT in intestinal folate transport is indisputable, the contribution of PCFT to placental folate transport is less clear. The extensive co-localization of placental PCFT with the major folate transporter FR α in the syncytiotrophoblast suggests a functional connection between PCFT and FR α -mediated folate uptake (6). However, unlike FR α and RFC, we did not observe a significant association between PCFT expression in the placenta and cord serum folate. Instead, our results suggest that PCFT may play a redundant role in placental folate transport, which is consistent with the undetectable PCFT-mediated folate transport in human syncytiotrophoblast at physiological pH (11) and with the normal phenotype of PCFT knockout mice at birth (10).

Because PCFT may function as a cellular heme transporter, we assessed its relationship with maternal and neonatal Fe status. Interestingly, placental PCFT was significantly positively associated with neonatal Fe status as evidenced by its association with cord hepcidin and EPO, which explained 18% and 12% variability in the mRNA and protein expression of placental PCFT, respectively. Similar associations were not evident between neonatal Fe status and FR α and RFC. The ability to completely correct the anemia phenotype by folate treatment (9, 10, 41) and the absence of Fe abnormalities other than anemia in human and animal models of PCFT deficiency strongly argue against an essential role of PCFT in systemic Fe homeostasis; however, these models were not specifically designed to assess placental and neonatal outcomes and thus it remains possible that PCFT may play a role in placental Fe utilization. It is unclear whether placental PCFT directly participates in heme uptake and/or intracellular heme trafficking as observed in human macrophages (42). The diffuse distribution of PCFT throughout the syncytiotrophoblast (6, 14) would support both speculations.

In summary, in a group of women with normal folate status and high prevalence of obesity, pre-pregnancy obesity had no impact on maternal folate levels in late gestation or neonatal folate status at birth. Consistent with a major role of FR α and RFC in placental folate transport, cord serum folate correlated positively with expression of both transporters in the placenta. In contrast, placental PCFT was not related to cord folate but showed a significant positive association with cord Fe status, which may suggest a role of PCFT in placental Fe transfer to the fetus.

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CHAPTER 4

MATERNAL RED BLOOD CELL CATABOLISM AS A SOURCE OF FETAL IRON⁵

Introduction

Data from National Health and Nutrition Examination Survey indicate that the prevalence of ID among US children aged 1-2 years has more than doubled over the past decade, increasing from 7% in 1999 to 15.9% in 2008 (1). The high prevalence of infant ID raises the concern that the birth Fe stores in some infants are inadequate to sustain growth and development over the first 6 months of life (2). The existence of critical periods in brain development during early infancy and the lack of early Fe screening programs for infants < 1 yr also point to the prenatal period as a critical window to minimize the negative impact of infant ID (3).

A human infant is born with 270 mg Fe, all of which is transported by the placenta from a combination of maternal absorption of dietary Fe, liver Fe stores, and RBC Hb Fe recycling (4). Relative utilization of these sources is unknown but data in Fe deficient rats suggest that 27% of fetal Fe is derived from maternal liver Fe stores and the remaining 73% is obtained from maternal Fe absorption and/or RBC Hb catabolism (5). It is estimated that diets with high Fe bioavailability contribute 3-4 mg Fe/day into the plasma Fe pool (6), but this amount is not sufficient to meet the 8-10 mg of Fe that are shuttled to the fetus daily over the last 4 weeks of pregnancy (7). Fe recycling from RBC Fe stores (20 mg Fe/day) provides the largest single Fe flux into the circulation (8), and thus may represent a significant Fe source for the fetus. The quantitative contribution of maternal RBC Fe to fetal Fe transfer has not been examined in humans. Whether placental transfer of this endogenous Fe can be up-regulated during maternal

⁵ Cao C, Pressman EK, Cooper EM, O'Brien. Results and Discussion to be written and manuscript to be submitted to the *FASEB Journal* in January 2015

Fe insufficiency similar to that which is known to occur at the level of the enterocyte for the absorption of dietary Fe (9) is unknown.

Hepcidin is a liver-derived hormone that integrates signals from body Fe stores, inflammation, and erythropoietic demand to control systemic Fe trafficking and tissue distribution (10). By binding and inducing the subsequent degradation of the Fe channel ferroportin, hepcidin decreases Fe entry into the circulation from duodenal Fe absorption, macrophage RBC Fe recycling, and hepatic Fe stores. Using a stable Fe isotope technique, we previously found that maternal hepcidin was inversely associated with intestinal non-heme Fe absorption (11) and placental transfer of dietary Fe (9). However, it is unclear whether placental transfer of Fe derived from maternal RBC stores is similarly regulated by maternal hepcidin concentrations.

Using a stable Fe isotope technique, we aimed to: 1) determine the magnitude of maternal RBC catabolism and relate this to maternal Fe status during pregnancy, 2) assess the relative contributions of maternal dietary Fe absorption in the third trimester of pregnancy and maternal RBC catabolism across pregnancy to net placental Fe transfer as measured by isotopic enrichment and Fe status in the neonate at birth, and 3) to characterize the role of maternal and neonatal hepcidin in determining placental transfer of Fe from the two maternal Fe sources.

Methods

Subjects

Pregnant participants (15-35 yrs) were recruited from the University of Rochester Midwifery Group and the Rochester Adolescent Maternity Program (RAMP) in Rochester, NY between 2012 and 2014. Pregnant volunteers were eligible to participate if they were healthy,

carrying a single fetus, and did not smoke cigarettes. Participants were excluded if they had gestational diabetes, hematological disorders, hypertension, or malabsorption diseases at the entry to the study. 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 participants. This trial was registered at clinicaltrials.gov as NCT01588665.

Study design and isotope dosing

Fe isotopes (^{57}Fe at 95% and ^{58}Fe at 93% enrichment) were purchased as metal from Trace Sciences International (Richmond Hill, Canada). The two tracers were converted into sterile, pyrogen-free solutions of ferrous sulfate using a 2:1 molar ratio of ascorbic acid: Fe following the procedure by Kastenmayer (12). Tracer solutions were tested for sterility and pyrogenicity (Analytical Research labs, AZ). Fe isotopic composition of the final tracer solutions was validated with the use of a ThermoQuest Triton TI Magnetic Sector Thermal Ionization Mass Spectrometer (TIMS, ThermoQuest Corporation, Bremen, Germany) and the total Fe content of the doses was measured using atomic absorption spectrophotometry (AAAnalyst 800; PerkinElmer Inc, Waltham, MA).

This study protocol required five study visits across gestation (**Figure 4.1**). At the first study visit at 15 weeks of gestation, fasting participants were asked to come to the RAMP clinic to consume a dose of 15.7 mg ^{57}Fe as ferrous sulfate (total Fe load of 16.6 mg) flavored with 3 mL of raspberry syrup (Humco, Texarkana, TX) administered orally by syringe. Participants were instructed to continue fasting for 2 hours after dosing and were then given a standard lunch to take home and consume. Because more than 80% of fetal Fe accumulation occurs over the second half of pregnancy in proportion to growing fetal weight (6), placental transfer of absorbed

^{57}Fe at gestational week 15 should be minimal (fetal Fe content from early whole body Fe analyses is estimated to be 20 mg at week 17 of gestation vs. 273 mg at week 34 of gestation) (13) and thus it is reasonable to assume that most of the ^{57}Fe recovered in cord blood is largely derived from endogenous maternal RBC Fe catabolism. Two weeks after ingesting the ^{57}Fe dose, women returned to the RAMP clinic for a collection of blood samples (10 mL) to determine RBC ^{57}Fe incorporation and Fe absorption. Whole blood samples (10 mL) were collected at following study visits to monitor changes in RBC ^{57}Fe enrichment that occurred as a consequence of RBC catabolism or due to the ~35% increase in RBC mass that occurs after the second trimester of gestation (**Figure 4.2**).

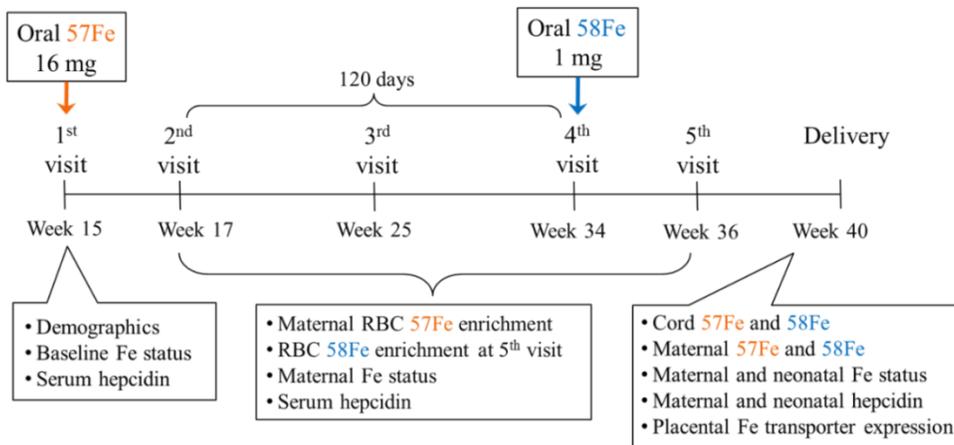


Figure 4.1. Study design and sample collection

Maternal body weight was recorded at each study visit (to the 0.1 g) using a calibrated scale. Once ^{57}Fe was incorporated into the RBC, the RBC ^{57}Fe content should remain relatively constant until the ^{57}Fe enriched RBCs reach the end of the RBC lifespan of 120 days (14, 15), corresponding with 34 weeks of gestation (**Figure 4.1**) or they may also decrease as the RBC

mass increases. The fourth study visit occurred at approximately week 34 of gestation. At this visit, women ingested a second oral stable Fe isotope dose (1.2 mg ^{58}Fe as ferrous sulfate) flavored with 1 mL of raspberry syrup (Humco) followed by 2 mL of a liquid Fe supplement (17.6 mg Fe) to provide a similar total Fe load to that obtained from the first stable Fe isotope dose. A blood sample was subsequently taken 2 weeks post-dosing to assess absorption of the second Fe isotope tracer. Participants were followed until delivery at which time a maternal blood sample (10 mL), a cord blood sample (30 mL) and samples of the placental tissue were collected.

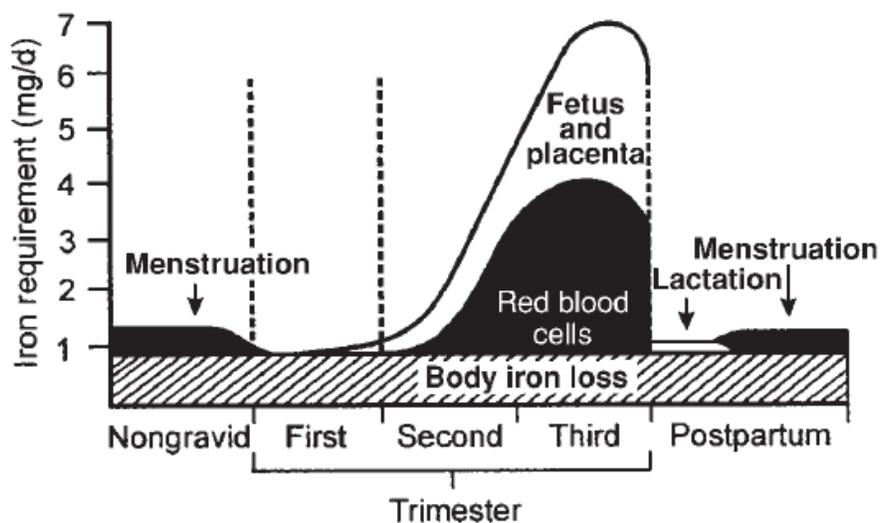


Figure 4.2. Estimated daily Fe requirements across gestation. Adopted from Bothwell (6).

Assessment of Fe status indicators

Whole blood Hb was measured using the HemoCue Hb 201 system (HemoCue, Brea, CA). Maternal anemia was defined as Hb < 11.0 g/dL in the first and third trimesters and Hb < 10.5 g/dL in the second trimester (16). Because Hb levels are lower in African American than

Caucasians with similar Fe status (17), Hb cutoffs for anemia were adjusted downward by 0.8 g/dL for African American women according to IOM recommendations (18). Neonatal anemia was defined as cord Hb concentrations < 13 g/dL (19). Serum was separated on site and shipped to Cornell on dry ice for analysis of Fe status indicators. SF and serum sTfR were measured with commercially available ELISA kits (Ramco Laboratories, Stafford, TX). TBI was calculated using the formula developed by Cook et al. (20): $TBI (mg/kg) = - [\log (\text{serum sTfR/SF}) - 2.8229]/0.1207$. Serum C-reactive protein (CRP), folate, vitamin B12, and erythropoietin (EPO) were measured using the Immulite 2000 immunoassay system (Siemens Medical Solutions, Malvern, PA). Serum hepcidin was measured using a commercially available Enzyme Immunoassay kit (s-1337; Bachem, San Carlos, CA) with a lower detection limit of 0.02 µg/L.

Determination of RBC isotopic enrichment

Whole blood samples (0.5 mL) collected at each study visit and cord blood obtained at delivery were digested with 4 mL Ultrex nitric acid in a polytetrafluoroethylene beaker. Samples were then dried on a hot plate and dissolved in 6N ultrapure hydrochloric acid (JT Baker, Phillipsburg, NJ). Iron was extracted with the use of anion exchange chromatography and was reconstituted in 3% nitric acid as previously described (9, 21).

Extracted Fe samples (8 µL) were loaded onto a rhenium filament (ultrapure rhenium obtained from H Cross Co, Weehawken, NY) along with 4 µL of silica gel (Sigma-Aldrich Inc, St Louis, MO) and 4 µL of phosphoric acid (0.7 N). Isotopic ratios of $^{57}\text{Fe}/^{56}\text{Fe}$ ($^{57/56}\text{Fe}$) and $^{58}\text{Fe}/^{56}\text{Fe}$ ($^{58/56}\text{Fe}$) were measured by TIMS and compared to the natural abundance values of $^{57/56}\text{Fe}$ (0.02317) and $^{58/56}\text{Fe}$ (0.00307) to generate delta percent excess values as previously reported (9, 11, 21). Relative SDs obtained averaged 0.026% and 0.24% for $^{57/56}\text{Fe}$ and $^{58/56}\text{Fe}$, respectively.

Calculation of Fe absorption and placental Fe transfer

Fe absorption was calculated using previously described methods assuming that 80% of absorbed Fe was incorporated into RBC within two weeks (11, 21, 22). The net amount of ^{57}Fe and ^{58}Fe incorporated in maternal RBC was determined by multiplying RBC enrichment of the two tracers by the total RBC Fe mass, which was estimated by using a blood volume for pregnant women (70 mL/kg), the Fe content of Hb (3.47 mg/g), subject's Hb value (g/dL), and weight (kg) (11, 23) using the following formula: Circulating Fe (mg) = 70 ml/kg \times weight (kg) \times Hb (g/dl) \times 0.01 \times 3.47 (mg/g).

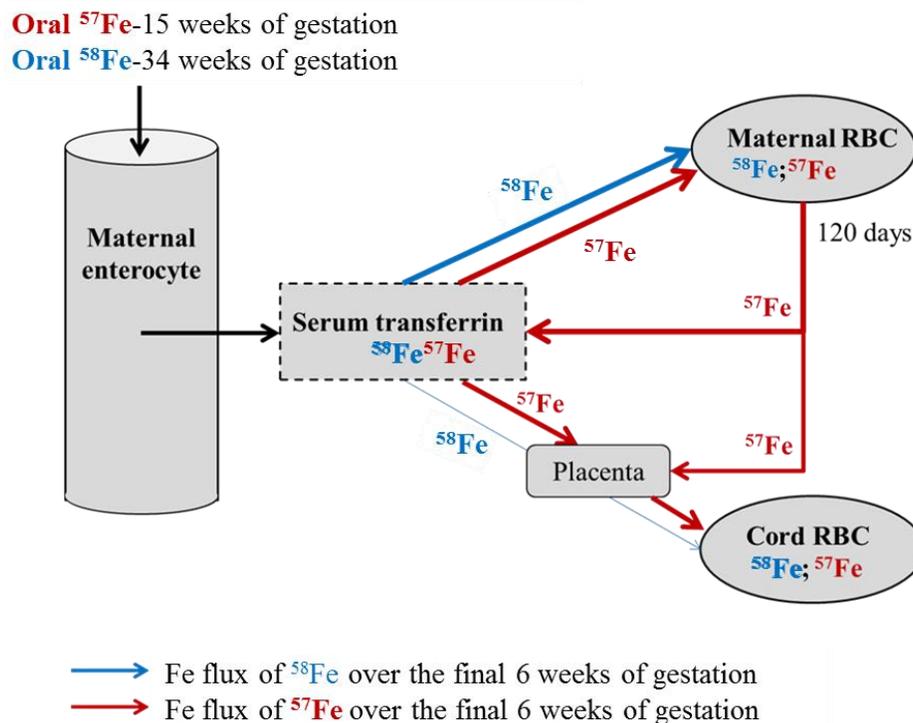


Figure 4.3. Schematic of *in vivo* Fe flux of oral stable Fe isotopes administered in the first (^{57}Fe) and third trimester (^{58}Fe) of pregnancy.

Net placental transfer of ^{57}Fe (derived from maternal RBC) and ^{58}Fe (derived from maternal diet) was estimated by measuring the total amount of ^{57}Fe and ^{58}Fe in the neonate at

birth. **Figure 4.3** is the diagram showing the placental transfer of the two oral tracers ingested at different time points during pregnancy. Total circulating Fe mass in the neonate was calculated, assuming a neonatal blood volume of 80 mL/kg (24, 25) and the Hb Fe content of 3.47 mg/g, with the following equation (9, 26): Circulating Fe pool (mg) = 80 mL/kg × birth weight (kg) × cord Hb (g/dL) × 3.47 (mg/g) × 0.01. The net amount of ⁵⁷Fe and ⁵⁸Fe present in neonatal circulation was determined by multiplying the total circulating Fe pool by the percent excess of ⁵⁷Fe and ⁵⁸Fe in cord blood, which reflects the degree to which the natural abundance ratio was increased as a result of fetal RBC incorporation of the tracer.

Statistical analysis

All statistical analyses were performed using JMP Pro10 (SAS Institute, Cary, NC). Difference between placental transfer of maternal endogenous ⁵⁷Fe and ⁵⁸Fe from recent absorption from the diet was analyzed using paired t-tests. Linear regression analysis was used to examine relationships between placental Fe transfer and Fe status indicators. Stepwise regression was used to identify significant determinants of placental transfer of Fe from maternal RBC (⁵⁷Fe) and diet (⁵⁸Fe). Values were presented as Mean ± SD. P-values < 0.05 were considered significant.

Preliminary results

Subject characteristics

General characteristics of the 16 study participants (15 adults and 1 adolescent) recruited to date are shown in **Table 4.1**. Average age at enrollment was 27.3 ± 4.1 y and more than half of the participants (56%) had a parity > 1 at entry into the study. Over 80% of the participants reported taking prenatal supplements daily at study enrollment. One subject was hospitalized for

deep vein thrombosis at gestational week 26 and was withdrawn from the study. Another subject developed preterm premature rupture of the membranes (PROM) at week 33 of gestation and therefore did not consume the second isotope dose scheduled at week 34. The 10 participants who have completed all study visits had term deliveries (≥ 37 weeks of gestation) and gave birth to normal weight infants (between 2,500 – 4,500 g).

Maternal and neonatal Fe status

Maternal anemia was evident in 9% of the participants at the first study visit (15 weeks of gestation) and was significantly higher at delivery with 27% of women having anemia (Hb < 11 g/dL). Cord Hb averaged 13.6 ± 2.9 g/dL and 50% (5/10) of the infants were anemic at birth. In the six participants with data on SF and sTfR across gestation, SF decreased by more than 50% from week 15 to week 36 of gestation (SF: 60.9 vs. 23.7 $\mu\text{g/L}$, $p=0.1$). A similar trend was evident for TBI across the same interval (8.2 vs. 3.9 mg/kg, $p=0.06$). Excluding the high cord SF concentration (752 $\mu\text{g/L}$) observed in one neonate that was treated for sepsis at birth, average cord SF concentrations were within published normative ranges (40-309 $\mu\text{g/L}$) for all other infants (27).

Table 4.1. Characteristics of study participants (n=16)

| Subject characteristics | Mean \pm SD (range) |
|---|------------------------------|
| Age (y) | 27.3 \pm 4.1 (17 – 34) |
| Parity | 1.0 \pm 1.2 (0 – 3) |
| Pre-pregnancy BMI (kg/m ²) | 25.1 \pm 7.9 (18.0 – 44.6) |
| Gestational age at delivery (wk) ¹ | 39.2 \pm 2.0 (34.7 – 41.4) |
| Race (%) | |
| Caucasian | 69 |
| African American | 31 |
| Ethnicity (%) | |
| Non-Hispanic | 88 |
| Hispanic | 12 |

¹Subjects who have delivered (n=12)

Maternal Fe absorption from the 17 mg dose of ^{57}Fe at week 15 of gestation was $11.1 \pm 9.4\%$ (n=12; range 0.8 – 27.6%) and was significantly correlated with serum sTfR ($r^2=0.53$, $p=0.01$) and TBI ($r^2=0.42$, $p=0.03$) but not with SF or hepcidin. Absorption of the second stable Fe isotope administered at week 34 of gestation was 2-times higher than Fe absorption at week 15 ($20.4 \pm 11.4\%$, $p=0.02$, n=7).

Placental transfer of maternal RBC- and diet -derived Fe

Average maternal enrichment of ^{57}Fe decreased across gestation from $2.6 \pm 1.7\%$ at week 17 of gestation to $2.0 \pm 1.2\%$ at delivery (n=6, paired t test=0.02).

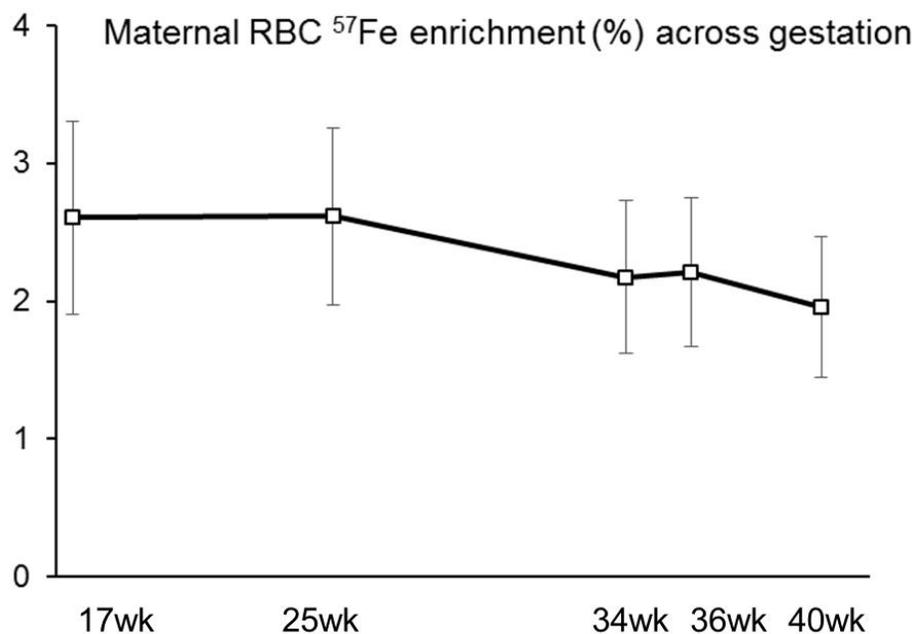


Figure 4.4. Mean changes in maternal RBC ^{57}Fe enrichment across gestation from 6 participants. Error bars represent SEMs.

In the eight neonates with complete isotope data, a total of 0.06 mg of ^{57}Fe and 0.02 mg of ^{58}Fe were present in the neonate at birth (**Table 4.2**).

Table 4.2. Placental transfer of oral stable Fe isotopes administered in the first (^{57}Fe) and third trimester of pregnancy (^{58}Fe)¹

| | ^{57}Fe (n=8) | ^{58}Fe (n=8) | <i>P</i> value |
|--|------------------------|------------------------|----------------|
| % excess in cord blood (dose adjusted) ² | 2.4 ± 1.4 | 7.1 ± 3.3 | 0.007 |
| Total tracer (mg) in cord blood | 0.06 ± 0.04 | 0.02 ± 0.01 | 0.01 |
| % of total recovered tracer (maternal RBC and neonatal RBC) present in neonate at birth ³ | 5.1 ± 1.7 | 5.8 ± 2.7 | 0.4 |

¹ Values are mean ± SD

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

³ Quantity of tracer in the neonate as a fraction of total tracer present in the maternal RBC and neonatal RBC at delivery

The net transfer of ^{57}Fe represented 5.1% of the total ^{57}Fe recovered in the maternal and neonatal RBC mass at delivery. The amount of ^{57}Fe in cord blood was positively correlated with the amount of ^{57}Fe incorporated in maternal RBC ($p=0.003$, $r^2=0.77$). In addition, the net transfer of ^{57}Fe to the fetus (mg) was significantly inversely associated with cord serum hepcidin ($p=0.01$, $r^2=0.65$), but not with maternal hepcidin ($p=0.7$). An inverse trend was also observed between net transfer of ^{58}Fe and cord hepcidin ($p=0.2$, $r^2=0.27$).

Preliminary conclusions and future plans

In a novel model of *in vivo* Fe flux across gestation, we demonstrated that stable Fe isotopes can be utilized to trace transfer of Fe to the fetus from the maternal RBC pool. A total of 16 subjects have been recruited to date. Preliminary results from 8 subjects with complete isotopic data have suggested that there is a large inter-subject variation in maternal RBC ^{57}Fe enrichment disappearance curves across gestation and it appears that placental Fe transfer is more closely related to cord serum hepcidin than maternal hepcidin. Isotopic analyses are ongoing to identify determinants of the slopes of RBC Fe enrichment across gestation.

To date, 12 of the 16 subjects have completed the study and the 16th subject recruited in April 2014 is due in October, 2014. Our study recruiter at the University of Rochester, Melissa Miller, will continue assisting with study visit scheduling and sample collection for the 4 participants who have yet to deliver their infants.

With regard to laboratory analyses, a total of 91 maternal and cord blood samples have been collected and more than half have been analyzed for RBC enrichment and serum Fe status indicators (SF and sTfR). More than 60% (24/38) of the serum samples collected from the mother at visit 1 and visit 4 and from the neonates at birth have been analyzed for serum hepcidin. Our lab manager, Tera Kent, will assist with laboratory analysis for the remainder of the study. We anticipate completing all laboratory work 2 months after the last participant has completed the study (i.e. by December, 2014) with a goal of submitting this final manuscript by January, 2015.

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CHAPTER 5

PRE-PREGNANCY BODY MASS INDEX AND GESTATIONAL WEIGHT GAIN HAVE A LIMITED IMPACT ON MATERNAL HEPcidIN BUT NO SIGNIFICANT IMPACT ON MATERNAL OR NEONATAL IRON STATUS⁶

Abstract

Obesity has been linked to suboptimal iron status in non-pregnant populations as a result of adiposity-associated inflammation increasing hepcidin production. During gestation, maternal obesity may negatively impact neonatal iron status. We aimed to assess the impact of maternal obesity and excessive gestational weight gain on maternal and neonatal iron status and to explore the possible mediating role of inflammation and estradiol on maternal and neonatal hepcidin. This study population included 230 pregnant adolescents (13-18 yrs) enrolled in either a longitudinal or a cross-sectional study. Pre-pregnancy BMI (ppBMI) and gestational weight gain (GWG) were obtained from medical records. Maternal iron status [hemoglobin, serum iron, ferritin, transferrin receptor, total body iron, and hepcidin], inflammation [interleukin-6, leptin] and serum estradiol were assessed at mid-gestation (26.2 ± 3.3 wks) in the longitudinal cohort and at delivery (39.8 ± 1.3 wks) in both study cohorts. Cord blood was collected in both studies and analyzed for iron indicators. Approximately 40% of the adolescents entered pregnancy overweight or obese. Multivariate analysis identified ppBMI as a predictor of serum iron at mid-gestation ($\beta = -0.026$, 95%CI: -0.05, -0.007; $p=0.009$) and serum hepcidin at delivery ($\beta=0.035$, 95%CI: 0.005, 0.06, $p=0.02$). None of the other maternal iron status indicators were significantly associated with ppBMI or GWG. Serum IL-6 was significantly associated with hepcidin at

⁶ Cao C, Pressman EK, Cooper EM, Guillet R, Westerman M, O'Brien KO. Submitted to *International Journal of Obesity* in June 2014

delivery ($\beta=0.23$, 95%CI: 0.1, 0.4, $p=0.0001$) but not at mid-gestation. Neither ppBMI nor GWG were significantly associated with neonatal Fe status indicators except for a positive relationship between ppBMI and cord Hb ($\beta=0.10$, 95%CI: 0.01, 0.2, $p=0.03$). These results suggest that adiposity-related inflammation does not override the iron mediated signals that regulate hepcidin production during pregnancy and in this adolescent cohort there is no strong evidence for a detrimental effect of maternal obesity and excessive weight gain on iron status in the offspring at birth.

Introduction

Approximately 60% of US women of reproductive age are overweight or obese (1) and women are gaining excessive weight across pregnancy (2). While excessive calorie intakes are common, iron deficiency (ID) remains a public health problem, with 30% of US pregnant women having depleted body iron (Fe) in the third trimester (3).

Epidemiological studies have long noted a link between overweight and low Fe status across different age groups (4). Research on the anemia of chronic disease suggests that this association may be mediated by an inflammation-associated increase in the cytokine interleukin 6 (IL-6), which directly stimulates liver production of the Fe regulatory hormone hepcidin (5). By inducing the degradation of the Fe exporter ferroportin (6), hepcidin inhibits Fe mobilization from intestinal Fe absorption, macrophage Fe recycling, and liver Fe stores, thus limiting Fe availability for tissue demands. In addition to IL-6, the adipokine leptin has been shown to stimulate hepcidin transcription *in vitro* and may represent another explanation for the association between adiposity and Fe status (7). Recently, estrogen has been shown to suppress hepcidin transcription by interacting with an estrogen response element in the hepcidin promoter (8). Because there is evidence for lower estrogen levels in obese pregnant women (9), reduced estrogen-mediated suppression of hepcidin may be another mechanism by which obesity increases hepcidin and negatively affects Fe status.

In support of the mediating role of inflammation in the obesity-hepcidin link, several studies in children and women showed higher circulating IL-6 and hepcidin in obese subjects compared with normal weight controls (10-12). It is unclear whether hepcidin responds similarly to adiposity-induced inflammation in pregnant women. A recent study by Dao et al. (4) suggests this may be the case. In 30 pregnant women with normal or obese pre-pregnancy BMI (ppBMI),

serum hepcidin was 2-times higher in those with obesity at entry into pregnancy compared with those that had a normal ppBMI. Interestingly, in the 13 women with available cord blood samples, ppBMI was inversely associated with cord serum Fe, an observation the authors attributed to impaired fetal Fe transfer in the obese mothers. However, serum Fe is not the most sensitive marker of Fe status and possible confounders such as dietary Fe intake and race were not considered in the analysis. A recent study in 316 pregnant women showed that maternal obesity was associated with lower neonatal SF and higher zinc protoporphyrin (13). These associations appeared to be largely driven by maternal diabetes as they were no longer significant in the non-diabetic mothers (n=234). In addition, this study did not measure hepcidin or IL-6/leptin, so it is unclear whether the observed association between maternal obesity and neonatal Fe status was mediated by hepcidin or was driven by diabetes-associated alterations in Fe homeostasis. Concurrent assessment of inflammation and hepcidin in healthy subjects are needed to clarify the relationship between obesity and Fe status in women with uncomplicated pregnancies.

In a large group of pregnant adolescents at risk for ID and obesity, we aimed to: 1) evaluate the impact of ppBMI and GWG on serum hepcidin and other Fe status indicators across gestation, 2) examine the relationships between IL-6, leptin, and estradiol with hepcidin across gestation, and 3) examine the relationships between ppBMI and GWG with neonatal hepcidin and Fe status at birth.

Methods

Study design and subjects

A total of 255 pregnant adolescents (13-18 yrs) were enrolled in two USDA-funded studies undertaken in Rochester NY between 2005 and 2012. The first study was designed to characterize longitudinal changes in maternal bone turnover in relation to fetal bone growth (14-18) and the second study aimed to examine the cross-sectional relationships between maternal and neonatal Fe status and is currently under review (19). Both studies collected maternal and cord blood at delivery and the longitudinal study collected an additional maternal blood sample at mid-gestation (26.2 ± 3.3 wks). The two study cohorts were recruited using similar eligibility criteria (age < 18 yrs, singleton pregnancy, no known medical conditions including diabetes and malabsorption diseases) and did not differ in ppBMI, GWG or maternal Fe status at delivery. One subject with biologically implausible weight loss from the self-reported pre-pregnancy weight to the medically obtained mid-gestation (14 kg) was excluded from all analyses. The final study population consisted of 230 pregnant adolescents in whom complete data on ppBMI, GWG, and Fe status assessment at mid-gestation (n=144) and/or at delivery (n=207) were available. Informed consent was obtained from all study participants and both studies were approved by the Institutional Review Boards at the University of Rochester and Cornell University.

Weight gain determinations

Pre-pregnancy BMI (kg/m^2) was calculated using pre-pregnancy weight and height recorded in the medical chart. BMI categories were defined in accordance with IOM guidelines as follows: underweight: BMI < 18.5; normal weight: BMI 18.5 – 24.9; overweight: BMI 25 – 29.9; or obese: BMI \geq 30. In some analyses, we further classified obesity into Class 1 (BMI 30 – 34.9), Class 2 (BMI 35 – 39.9), and Class 3 (BMI \geq 40).(20) Due to the small number of participants with Class 3 obesity, Class 2 and 3 obesity were collapsed into one group. Total

GWG was calculated as the difference between pre-pregnancy weight and weight recorded by clinical staff at delivery. Adequate total GWG was defined as a total weight gain of 12.5 – 18 kg for underweight women, 11.5 – 16 kg for normal weight women, 7 – 11.5 kg for overweight women, and 5 – 9 kg for obese women.(2) For participants in the longitudinal cohort, weight data were also available at mid-gestation corresponding to the time when the maternal blood samples were obtained. We determined the range of adequate weight gain specific to the gestational week at mid-gestation using ppBMI-specific recommendations for first trimester and total gestational weight gain according the method by Chmitorz et al.(21)

Biochemical analyses

All whole blood samples were assessed for hemoglobin (Hb) by the hospital clinical laboratory as previously reported (22). Serum was separated and stored at -80°C prior to analysis. Serum ferritin (SF) and soluble transferrin receptor (sTfR) were assessed using ELISA kits (Ramco Laboratories, Stafford, TX). Maternal total body iron (TBI) was calculated from SF and sTfR using the equation by Cook et al.(23)

Maternal anemia in the Caucasian adolescents was defined as Hb values < 10.5 g/dL in the second trimester, or < 11.0 g/dL in third trimester (24). Because the Hb distribution for African Americans is shifted to the left of that for the Caucasians, we adjusted the Hb cutoffs downward by 0.8 g/dL to define anemia in African American teens as recommended by IOM for black women (25). Specifically, anemia in African American teens was defined as Hb < 9.7 g/dL in the second and Hb < 10.2 g/dL in the third trimester. Maternal ID was defined cutoffs validated in pregnancy women as either SF < 12 $\mu\text{g/L}$ (26), serum sTfR > 8.5 mg/L (27), or TBI < 0 mg/kg (26). Neonatal anemia was defined as cord Hb < 13 g/dL (28). Neonatal total body iron (TBI) was calculated using the formula developed in healthy infants (29, 30): TBI (mg) =

Hb Fe [body weight (kg) × Hb × 2.74] + storage Fe [21.99 × log (SF) -29.04] + functional tissue Fe [body weight (kg) × 7 mg/kg].

Serum erythropoietin (EPO) was measured by the Immulite 2000 immunoassay system (Seimens, Los Angeles, CA). Serum hepcidin was measured by Intrinsic LifeSciences (La Jolla, CA) using a competitive ELISA with a lower limit of detection of 5 µg/L (31). Hepcidin values below the detection limit were assigned a value of 2.5 µg/L for data analysis purposes as previously reported (32). Serum Fe was measured by graphic furnace atomic absorption spectrophotometry (Perkin Elmer AAnalyst 800, Norwalk, CT) in samples without visual signs of hemolysis (81.8% of total available). Serum IL-6 was measured with a multiplex assay kit (Millipore, Billerica, MA). Serum C-reactive protein (CRP) was measured in mid-gestation samples only using Immulite 2000 immunosystem (Seimens). Serum leptin was analyzed by ELISA (Millipore, Billerica, MA). Estradiol was analyzed using ELISA (Alpco Diagnostics, Salem, NH).

Questionnaires about dietary Fe intake

Using health questionnaire data obtained during the third trimester of pregnancy, participants from both studies self-reported the frequency of prenatal supplement use and their current smoking status. Dietary Fe intake was estimated by 24-h dietary recall in both studies by a registered dietitian using the Nutrition Data System for Research (University of Minnesota, Minneapolis, MN).

Statistical analyses

Data were analyzed using JMP Pro10 (SAS Institute, Cary, NC). Two adolescents self-identified as American Indian. Excluding these two subjects did not change any of the study results and none of the race-specific analyses differed if these two adolescents were grouped with

either the African American or Caucasian cohort. To meet the requirement of statistical assumptions and power considerations, data from the two adolescents were included within the African American race cohort. Variables with skewed distributions were log-transformed (except for cord hepcidin and maternal estradiol which were transformed using square root) before analysis. Differences in biochemical measures between the ppBMI and GWG groups were assessed by ANOVA and Tukey Post-hoc tests. Bivariate correlations were analyzed by Pearson's correlation. The relationships between ppBMI and GWG with Fe status were also analyzed in multivariate models controlling for maternal age, race, gestational age at blood draw, and dietary Fe intake. For models of maternal hepcidin at delivery, delivery mode (vaginal and cesarean) was also included as a previous study showed differences in maternal hepcidin by mode of delivery (33). Data were reported as mean \pm SD. P values < 0.05 were considered significant.

Results

Demographic characteristics of the study population (n=230) stratified by ppBMI are presented in **Table 5.1**. Average age of the adolescents at enrollment was 17.2 ± 1.1 yrs and about 37.8% entered pregnancy overweight or obese. Among the 41 obese teens, thirteen had Class 2 obesity ($BMI \geq 35 \text{ kg/m}^2$). A smaller percentage of overweight or obese teens had a spontaneous vaginal delivery when compared to the underweight or normal weight teens (82.6% vs. 91.3%, $p=0.051$). More than half of the obese adolescents (58.3%) gained excessive weight by mid-gestation and 92.5% exhibited excessive GWG at delivery. Among the 227 adolescents with data on glucose tolerance test, one normal weight subject had a confirmed diagnosis of gestational diabetes at 30 weeks of gestation. Excluding this subject did not change any of the

study results. There were no differences in race, ethnicity, smoking status, prenatal supplement use, or daily dietary Fe intake across ppBMI categories.

Relationships between ppBMI and weight gain with inflammation across gestation

We examined ppBMI both as a categorical and a continuous variable in relation to cytokines and estradiol levels across gestation. At mid-gestation, approximately 40.9% of the teens had elevated CRP (> 5 mg/L). Serum leptin, CRP, and IL-6 were more than 2-times higher in obese compared with normal weight teens (**Table 5.1**) and showed significant positive inter-correlations ($p < 0.05$ for all correlations). At delivery, serum leptin remained elevated in the overweight and obese groups but IL-6 in the obese teens no longer differed from the normal weight group. Bivariate analyses showed that ppBMI was positively associated with serum leptin ($r = 0.58$, $p < 0.0001$), CRP ($r = 0.50$, $p < 0.0001$), and IL-6 ($r = 0.24$, $p = 0.004$) at mid-gestation. Serum estradiol at mid-gestation did not differ significantly between ppBMI groups but there was a trend for an inverse correlation between ppBMI and estradiol ($p = 0.1$). At delivery, ppBMI was correlated with serum leptin ($r = 0.48$, $p < 0.0001$) but not with IL-6 or estradiol.

We also compared inflammatory markers and estradiol levels between weight gain categories. At mid-gestation, serum leptin but not IL-6 was significantly higher in teens who gained above the recommended weight gain range compared with those gaining within or below the range. At delivery, leptin remained elevated in teens with excessive GWG but there was no difference in IL-6 between GWG groups. There was no difference in serum estradiol among weight gain categories at mid-gestation or at delivery.

Table 5.1. Characteristics of study participants by pre-pregnancy BMI category¹

| Characteristics | Pre-pregnancy BMI (kg/m ²) | | | No. of subjects | |
|---|--|----------------------------|--------------------------|--------------------------|-----------------|
| | Underweight < 18.5 | Normal weight 18.5-24.9 | Overweight 25.0-29.9 | | Obese ≥ 30.0 |
| Subjects [n (%)] | 16 (7.0) | 127 (55.2) | 46 (20.0) | 41 (17.8) | 230 |
| Age at enrollment (y) | 17.0 ± 1.1 | 17.1 ± 1.0 | 17.1 ± 1.2 | 17.4 ± 1.0 | 230 |
| Pre-pregnancy BMI (kg/m ²) | 17.5 ± 0.8 ^a | 21.9 ± 1.8 ^b | 27.4 ± 1.4 ^c | 34.0 ± 4.0 ^d | 230 |
| Parity > 0 (%) | 12.5 | 15.0 | 13.0 | 26.8 | 230 |
| Smoking during pregnancy (%) | 6.3 | 8.0 | 4.4 | 4.9 | 227 |
| Gestational age at mid-gestation (wk) | 26.4 ± 3.6 | 26.2 ± 3.3 | 26.9 ± 3.2 | 25.4 ± 3.5 | 144 |
| Gestational age at delivery (wk) | 38.4 ± 3.1 | 39.1 ± 2.4 | 39.9 ± 2.2 | 39.8 ± 3.2 | 227 |
| Preterm delivery (%) | 18.8 | 8.9 | 4.4 | 2.4 | 227 |
| Delivery mode (%) | | | | | 224 |
| Vaginal delivery | 93.8 | 91.0 | 78.3 | 87.5 | |
| Daily prenatal supplement use (%) | 50.0 | 39.5 | 60.9 | 45.0 | 226 |
| Dietary Fe intake (mg/d) | 21.6 ± 24.0 | 17.5 ± 7.7 | 15.0 ± 6.5 | 16.6 ± 7.8 | 216 |
| Race (%) | | | | | 230 |
| African American | 68.7 | 70.9 | 73.9 | 65.5 | |
| Ethnicity (%) | | | | | 230 |
| Non-Hispanic | 81.2 | 74.8 | 78.3 | 65.8 | |
| Weight gain adequacy at mid-gestation (%) ² | | | | | 144 |
| Inadequate | 30.0 | 36.6 | 14.3 | 29.2 | |
| Adequate | 30.0 | 31.7 | 17.9 | 12.5 | |
| Excessive | 40.0 | 31.7 | 67.9 | 58.3 | |
| Total gestational weight gain adequacy (%) ² | | | | | 224 |
| Inadequate | 26.7 | 19.5 | 8.7 | 0.0 | |
| Adequate | 26.7 | 29.3 | 15.2 | 7.5 | |
| Excessive | 46.7 | 51.2 | 79.1 | 92.5 | |
| Infant weight (kg) | 2.98 ± 0.79 ^a | 3.12 ± 0.49 ^a | 3.42 ± 0.49 ^b | 3.40 ± 0.45 ^b | 223 |

¹ Values are means ± SDs. Means in rows with different letters are significantly different

² Group differences were significant, P < 0.05 (Chi-square test)

Relationships between ppBMI and weight gain with maternal Fe status across gestation

Maternal Fe status indicators and hepcidin by ppBMI categories are shown in **Table 5.2**.

None of the maternal Fe status indicators at mid-gestation or at delivery differed significantly among ppBMI categories. Teens gaining lower than recommended GWG had lower hepcidin at delivery compared with those who gained within or greater than recommendation.

Table 5.2. Pre-pregnancy BMI and maternal and neonatal Fe status indicators, inflammation markers, and estradiol concentrations¹

| | Pre-pregnancy BMI | | | |
|---------------------------|---------------------------|----------------------------|--------------------------|--------------------------|
| | Underweight < 18.5 | Normal weight 18.5-24.9 | Overweight 25.0-29.9 | Obese ≥ 30 |
| Maternal | | | | |
| Mid-gestation | | | | |
| Sample size (n) | 10 | 82 | 28 | 24 |
| Hemoglobin (g/dL) | 10.9 ± 1.2 | 11.1 ± 0.9 | 11.3 ± 0.8 | 11.4 ± 0.8 |
| Anemia (%) | 22.2 | 8.1 | 0.0 | 9.5 |
| Serum ferritin (µg/L) | 14.8 ± 7.5 | 22.4 ± 17.8 | 24.3 ± 16.6 | 28.4 ± 25.5 |
| < 12 (%) | 30.0 | 31.7 | 17.9 | 20.8 |
| Serum sTfR (mg/L) | 5.6 ± 2.5 | 5.1 ± 3.6 | 5.2 ± 6.8 | 4.9 ± 2.2 |
| > 8.5 (%) | 10.0 | 6.1 | 3.6 | 12.5 |
| Total body Fe (mg/kg) | 1.5 ± 4.4 | 3.3 ± 3.7 | 4.3 ± 3.5 | 3.7 ± 5.0 |
| < 0 (%) | 20.0 | 14.6 | 10.7 | 20.8 |
| Serum Fe (µg/dL) | 94 ± 49 ^{a,b} | 127 ± 87 ^a | 97 ± 40 ^{a,b} | 77 ± 32 ^b |
| Serum EPO (mIU/mL) | 38.3 ± 34.9 | 31.7 ± 20.7 | 30.7 ± 12.0 | 36.7 ± 24.0 |
| Serum hepcidin (µg/L) | 18.7 ± 24.6 | 27.3 ± 25.1 | 35.6 ± 32.1 | 29.8 ± 19.3 |
| < 5 (%) | 10.0 | 6.2 | 3.7 | 0 |
| C-reactive protein (µg/L) | 2.0 ± 1.1 ^a | 4.2 ± 4.4 ^a | 10.7 ± 8.7 ^b | 9.0 ± 5.3 ^b |
| > 5 (%) | 0.0 | 27.9 | 66.7 | 70.0 |
| Serum leptin (µg/L) | 17.9 ± 8.2 ^{a,b} | 20.3 ± 12.7 ^a | 34.4 ± 18.6 ^b | 54.9 ± 24.1 ^c |
| Serum IL-6 (µg/L) | 1.2 ± 1.2 | 3.3 ± 8.9 | 5.1 ± 10.3 | 7.0 ± 22.0 |
| Serum estradiol (pg/mL) | 3255 ± 1743 | 3199 ± 1775 | 3273 ± 1866 | 2504 ± 1020 |
| Delivery | | | | |
| Sample size (n) | 14 | 109 | 43 | 41 |
| Hemoglobin (g/dL) | 10.9 ± 1.0 | 11.6 ± 1.5 | 11.4 ± 1.3 | 11.5 ± 1.4 |
| Anemia (%) | 21.4 | 20.6 | 14.6 | 17.1 |
| Serum ferritin (µg/L) | 37.6 ± 69.8 | 30.7 ± 30.2 | 26.8 ± 30.9 | 29.9 ± 18.6 |
| < 12 (%) | 28.6 | 20.6 | 25.6 | 17.1 |
| Serum sTfR (mg/L) | 6.7 ± 3.6 | 5.6 ± 2.9 | 5.1 ± 2.2 | 5.7 ± 4.4 |
| > 8.5 (%) | 21.4 | 12.8 | 9.3 | 19.5 |
| Total body Fe (mg/kg) | 2.6 ± 5.4 | 3.9 ± 4.1 | 3.6 ± 3.5 | 4.4 ± 3.9 |

Table 5.2. (Continued)

| | | | | |
|------------------------------------|---------------------------------|--------------------------------|---------------------------------|-------------------------------|
| < 0 (%) | 28.6 | 15.0 | 14.0 | 9.8 |
| Serum Fe ($\mu\text{g/dL}$) | 88.9 \pm 43.4 | 135.3 \pm 149.0 | 104.4 \pm 67.7 | 107.8 \pm 67.7 |
| Serum EPO (mIU/mL) | 39.8 \pm 44.9 | 33.0 \pm 24.7 | 36.3 \pm 31.1 | 28.8 \pm 23.9 |
| Serum hepcidin ($\mu\text{g/L}$) | 21.5 \pm 18.4 | 38.5 \pm 42.0 | 42.0 \pm 41.6 | 44.5 \pm 33.3 |
| < 5 (%) | 7.7 | 13.9 | 4.7 | 5.0 |
| Serum leptin ($\mu\text{g/L}$) | 23.4 \pm 13.9 ^a | 29.0 \pm 18.6 ^{a,b} | 41.6 \pm 23.0 ^b | 57.8 \pm 23.7 ^b |
| Serum IL-6 ($\mu\text{g/L}$) | 5.4 \pm 5.2 | 10.4 \pm 19.5 | 10.4 \pm 14.3 | 5.8 \pm 8.8 |
| Serum estradiol (pg/mL) | 4713 \pm 2162 | 5688 \pm 3086 | 6895 \pm 2881 | 5446 \pm 2376 |
| Neonatal | | | | |
| Sample size (n) | 14 | 101 | 40 | 36 |
| Hemoglobin (g/dL) | 13.4 \pm 2.4 | 14.0 \pm 2.8 | 14.0 \pm 2.3 | 15.5 \pm 2.4 |
| Anemia (%) | 45.5 | 27.4 | 18.5 | 9.5 |
| Serum ferritin ($\mu\text{g/L}$) | 198.2 \pm 179.0 | 147.4 \pm 94.7 | 133.3 \pm 105.9 | 144.6 \pm 95.5 |
| < 76 (%) | 21.4 | 19.2 | 40.0 | 25.0 |
| Serum sTfR (mg/L) | 6.9 \pm 2.6 | 7.9 \pm 2.9 | 9.0 \pm 4.2 | 8.7 \pm 4.7 |
| Body Fe content (mg) | 156.0 \pm 34.5 ^{a,b} | 160.2 \pm 31.2 ^a | 174.8 \pm 28.8 ^{a,b} | 184.0 \pm 26.6 ^b |
| Serum Fe ($\mu\text{g/dL}$) | 252.2 \pm 95.5 | 221.9 \pm 110.0 | 193.1 \pm 60.2 | 207.0 \pm 60.0 |
| Serum EPO (mIU/mL) | 42.6 \pm 35.7 | 41.0 \pm 42.8 | 194.0 \pm 862.1 | 54.1 \pm 55.3 |
| Serum hepcidin ($\mu\text{g/L}$) | 73.9 \pm 48.5 | 121.5 \pm 84.2 | 144.9 \pm 121.2 | 123.0 \pm 76.4 |

¹ Values are mean \pm SD, or %; Means in rows with different letters are significantly different

There was no difference in the prevalence of anemia and ID between ppBMI or GWG groups. Further dividing obesity categories into Class 1 (ppBMI: 30-35) and Class 2 or above (ppBMI \geq 35) revealed that serum hepcidin at mid-gestation in adolescents with Class 2 and 3 obesity was 1.5-times higher than the normal weight group (39.6 vs. 27.3 $\mu\text{g/L}$, $p=0.1$). Similar to hepcidin, SF at mid-gestation was significantly elevated in teens with ppBMI \geq 35 compared with the normal weight teens (46.0 vs. 22.4 $\mu\text{g/L}$, $p=0.02$).

Possible relationships were explored with ppBMI and weight gain as continuous traits in relation to Fe status and hepcidin across gestation. There was a weak positive correlation between ppBMI and serum hepcidin at mid-gestation ($r=0.17$, $p=0.04$; **Figure 5.1**) but this relationship lost significance after controlling for maternal age, race, gestational age at mid-gestation, weight gain at mid-gestation, and dietary Fe intake (**Table 5.3**). Serum hepcidin at delivery was significantly correlated with ppBMI ($r=0.19$, $p=0.006$; **Figure 5.1**) and ppBMI

remained a significant predictor of maternal hepcidin at delivery after adjusting for age, race, gestational age at delivery, delivery mode, and dietary Fe intake (**Table 5.3**). Serum Fe at mid-gestation was inversely associated with ppBMI both in bivariate analysis ($r = -0.21$, $p = 0.02$) and in the adjusted model (**Table 5.3**). No relationship was evident between weight gain and maternal Fe status except for a trend for a positive correlation between GWG and serum EPO at delivery ($r = 0.12$, $p = 0.08$).

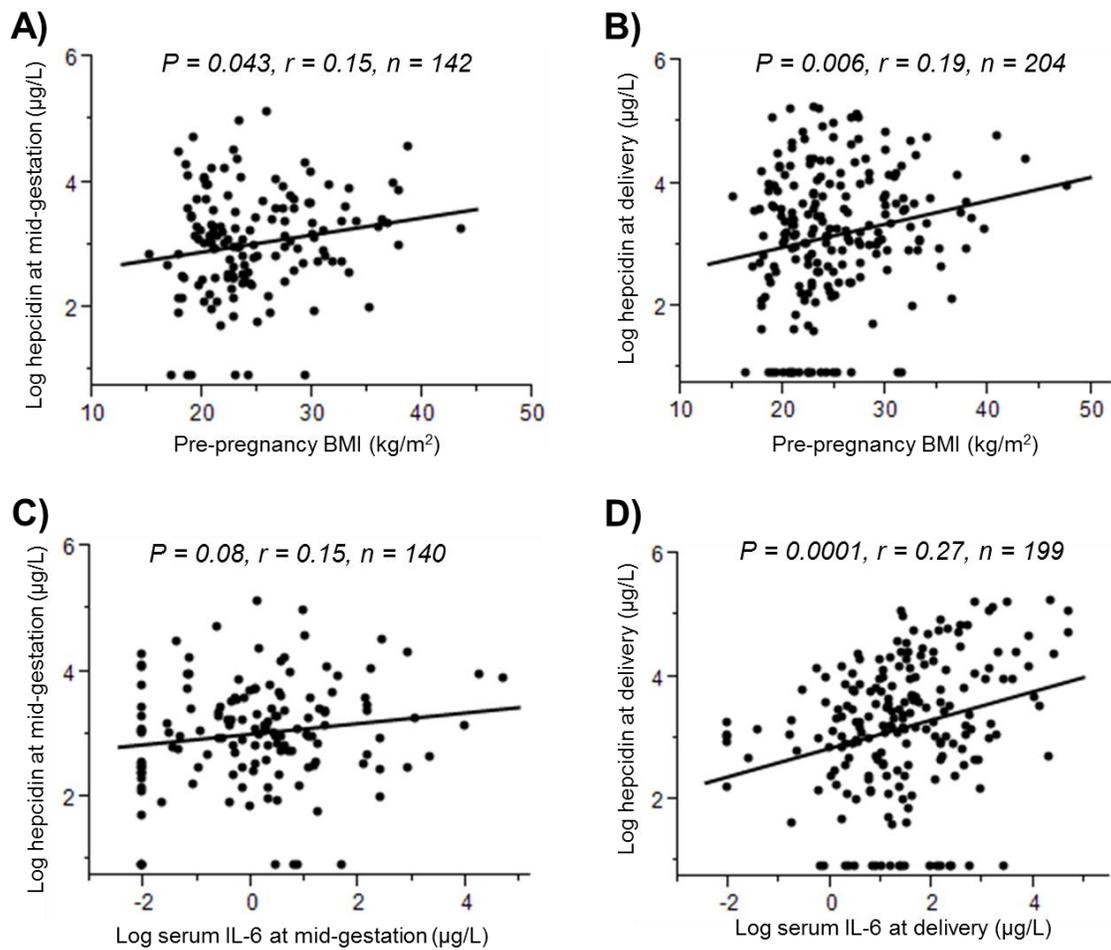


Figure 5.1. Correlations between ppBMI and serum IL-6 with hepcidin. ppBMI was positively associated with A) serum hepcidin at mid-gestation; and B) serum hepcidin at delivery. C) Serum IL-6 was insignificantly correlated with hepcidin at mid-gestation but was significantly correlated with hepcidin at delivery (D).

Table 5.3. Significant effects of pre-pregnancy BMI and weight gain on maternal Fe status indicators

| Variables | β | 95% CI | P-value | R ² model |
|--|---------|---------------|---------|----------------------|
| Mid-gestation serum Fe ¹ (n=123) | | | | 0.09 |
| Pre-pregnancy BMI (kg/m ²) | -0.026 | -0.05,-0.007 | 0.009 | |
| Weight gain at mid-gestation (kg) | -0.005 | -0.02,0.01 | 0.58 | |
| Mid-gestation hepcidin ¹ (n=141) | | | | 0.07 |
| Pre-pregnancy BMI (kg/m ²) | 0.026 | -0.0004, 0.05 | 0.054 | |
| Weight gain at mid-gestation (kg) | -0.010 | -0.04, 0.02 | 0.49 | |
| Delivery serum hepcidin ² (n=191) | | | | 0.06 |
| Pre-pregnancy BMI (kg/m ²) | 0.035 | 0.005, 0.06 | 0.024 | |
| Total gestational weight gain (kg) | 0.0096 | -0.01, 0.03 | 0.38 | |

¹ Adjusted for maternal age, race, gestational age at mid-gestation, and dietary Fe intake during pregnancy

² Adjusted for maternal age, race, gestational age at delivery, dietary Fe intake during pregnancy, and delivery mode

Relationships between hepcidin and IL-6, leptin, and estradiol across gestation

To explore a possible mediating role of inflammation and estrogen in the relationship between ppBMI and hepcidin, we analyzed the relationships between serum IL-6, leptin, and estradiol with hepcidin across pregnancy. At mid-gestation, there was a trend for a positive correlation between IL-6 and hepcidin ($r=0.15$, $p=0.08$; **Figure 5.1**). Leptin was not associated with hepcidin ($p=0.9$). Serum estradiol was insignificantly inversely correlated with hepcidin ($r=-0.16$, $p=0.06$). At delivery, neither serum leptin nor estradiol was related to hepcidin. Serum IL-6 was positively correlated with hepcidin ($r=0.27$, $p=0.0001$; **Figure 5.1**). Excluding subjects with undetectable hepcidin at delivery further strengthened the correlation between hepcidin and IL-6 ($r=0.37$, $p<0.0001$). Serum IL-6 remained a significant predictor of hepcidin in multivariate analysis controlling for age, race, gestational age at delivery, and delivery mode ($\beta=0.23$,

p=0.0001, $r^2 = 0.09$). A model that included SF, EPO, and IL-6 at delivery explained 22% of the variation in hepcidin at delivery.

Relations between ppBMI and GWG with neonatal hepcidin and Fe status

Neonatal Fe status indicators in each maternal ppBMI category are presented in **Table 5.2**. Anemia was evident in 24% of the newborns at birth and 25% of the neonates had SF < 76 $\mu\text{g/L}$, a cutoff that was previously associated with poor psychomotor performance at 5 years of age (34). Infants born to obese mothers had greater body Fe content than those born to normal weight mothers (184.0 vs. 160.2 mg, p=0.01). Neonates born to adolescents in higher ppBMI categories had higher cord Hb (p for trend=0.03). None of the other neonatal Fe status indicators including hepcidin differed among maternal ppBMI groups. With the exception of body Fe content, none of the neonatal Fe status indicators showed significance differences between teens who gained excess weight compared to those who gained within the normal range.

Table 5.4. Associations between neonatal Fe status indicators and maternal ppBMI and GWG

| Neonatal Fe status ¹ | ppBMI (kg/m^2) | | | GWG (kg) | | |
|------------------------------------|---------------------------|---------------|-------|----------|---------------|-------|
| | β | 95% CI | P | β | 95% CI | P |
| Hb (g/dL) | 0.10 | 0.01, 0.19 | 0.028 | -0.0030 | -0.07, 0.06 | 0.93 |
| SF ($\mu\text{g/L}$) | -0.011 | -0.03, 0.01 | 0.33 | 0.0026 | -0.01, 0.02 | 0.76 |
| Serum sTfR (mg/L) | 0.0092 | -0.0005, 0.02 | 0.064 | 0.0016 | -0.006, 0.009 | 0.67 |
| Body Fe content (mg) | 0.76 | -0.05, 1.6 | 0.065 | -0.019 | -0.6, 0.6 | 0.95 |
| Serum hepcidin ($\mu\text{g/L}$) | 0.036 | -0.07, 0.14 | 0.51 | -0.073 | -0.15, 0.006 | 0.070 |
| Serum Fe ($\mu\text{g/dL}$) | -0.0045 | -0.02, 0.010 | 0.54 | -0.0021 | -0.01, 0.009 | 0.70 |
| Serum EPO (mIU/mL) | -0.0024 | -0.02, 0.03 | 0.86 | 0.0048 | -0.01, 0.02 | 0.61 |

¹ Adjusted for infant sex, birth weight, and gestational age at delivery

Multivariate regression was used to assess the influence of ppBMI and GWG on neonatal Fe status controlling for infant weight, sex, and gestational age at birth (**Table 5.4**). Neither

ppBMI nor GWG were significantly associated with cord SF, sTfR, serum Fe, EPO, body Fe content, or hepcidin. Higher GWG tended to be associated with lower cord hepcidin ($\beta = -0.073$, $p = 0.07$). Similar to the finding in categorical analysis, ppBMI was a positive predictor of cord Hb ($\beta = 0.10$, $p = 0.03$).

Discussion

To our knowledge, this is the first study to examine the impact of both maternal ppBMI and GWG on hepcidin and inflammatory markers in the mother and her neonate in a large cohort of pregnant adolescents across gestation. Concurrent assessment of IL-6, leptin, and estradiol provided a unique opportunity to explore possible mediating factors in the relationship between obesity and Fe status. We report for the first time different relationships between weight, IL-6, and hepcidin at mid-gestation compared to those at delivery. Maternal obesity was associated with elevated serum IL-6 but not hepcidin at mid-gestation. Serum IL-6 at delivery was on average 2.2-times higher than mid-gestation (9.1 vs. 4.1 $\mu\text{g/L}$, $p < 0.0001$) and was positively correlated with serum hepcidin at delivery. With the exception of serum Fe at mid-gestation, maternal obesity and excessive GWG did not negatively impact maternal Fe status during pregnancy. None of the neonatal Fe status indicators were adversely impacted by maternal obesity and excessive GWG and higher ppBMI was associated with higher cord Hb. SF is the most significant determinant of maternal hepcidin both at mid-gestation and delivery. Collectively, our results suggest that adiposity-associated inflammation does not override the impact of low Fe status in the regulation of hepcidin production during pregnancy and there is little evidence for a detrimental effect of maternal obesity and excessive weight gain on Fe status in the offspring at birth.

Studies in non-pregnant women (n=40) (12) and pregnant adults (n=29) (4) demonstrated a 9- and 2-times greater serum hepcidin, respectively, in the obese than normal weight group. In our study, serum hepcidin in obese pregnant adolescents was not significantly elevated compared with the normal weight group. This discrepancy may reflect differences in degree of adiposity of the different study populations. Average BMIs of the obese participants in previous studies were above the cutoff for class 2 obesity ($BMI \geq 35$) while the majority of obese teens in our study had Class 1 obesity. When the obese group in our study was further stratified into different classes of obesity, we observed a 50% increase in hepcidin in adolescents with Class 2 and above obesity compared with the lean group. Although the difference in hepcidin between the two groups did not reach statistical significance, possibly due to small number of teens in the extreme obese category, our data are consistent with previous studies that showed higher hepcidin in obese individuals with extremely high BMI and support the notion that a certain level of adiposity may be necessary to elicit a significant elevation in hepcidin (35, 36). The increase in hepcidin in the high BMI group did not appear to be mediated by IL-6 or leptin, as neither was associated with hepcidin at mid-gestation and IL-6 was not elevated compared with the normal weight adolescents. Interestingly, the most obese adolescents also had the lowest estradiol levels, which may have contributed to higher hepcidin in the extreme obese group. This, and the trend for an inverse correlation between serum estradiol and hepcidin at mid-gestation, supports the cell culture and animal data demonstrating a suppressive effect of estrogen on hepcidin transcription (8). It is possible that other hepcidin regulators, such as bone morphogenetic protein 6 (37), IL-22 (38), and activin B (39), also contributed to the hepcidin increase in adolescents with a high degree of obesity. The significance of these potential hepcidin modulators in severely obese populations warrants further investigation.

A negative association between ppBMI and serum Fe was evident at mid-gestation, which is consistent with data in women and female adolescents (40-43). The link between low serum Fe and adiposity is often attributed to inflammation-induced increases in hepcidin, but the lack of association between hepcidin and serum Fe in our study and others (44-46) does not support this explanation. An alternative explanation for obesity-associated hypoferremia has been proposed (40, 42) and involves lipocalin-2, an Fe binding protein that is highly expressed in adipose tissue (47, 48). Adipose lipocalin-2 increases in obesity (49), and thus may lead to Fe sequestration in adipose tissue. Future studies are needed to test this hypothetical role of lipocalin-2 and examine lipocalin-2 expression in relation to serum Fe.

We assessed a potential mediating role of inflammation in the link between weight and hepcidin by measuring both IL-6 and leptin, cytokines that have been shown to stimulate hepcidin transcription *in vitro* (5, 7). Consistent with increased inflammation in the obese state (50, 51), concentrations of IL-6, leptin, and CRP at mid-gestation were significantly elevated in the obese adolescents compared with the lean group. Contrary to the *in vitro* data, neither IL-6 nor leptin were correlated with hepcidin at mid-gestation. A positive correlation between IL-6 and hepcidin has been reported in patients with Hodgkin's lymphoma (52), end-stage renal disease (53), and sepsis (54), but not in healthy pre-menopausal women (12) or pregnant women (4, 46). A potent immune-modulatory cytokine, IL-6 is elevated in many diseases and inflammatory conditions (55). The absence of a positive relationship between serum IL-6 and hepcidin in individuals without severe inflammation may suggest that a threshold level of IL-6 must be reached to significantly impact hepcidin production. Due to the narrow range of IL-6 concentrations evident in our population, we were not powered to determine such a threshold. Nevertheless, the positive correlation between IL-6 and hepcidin at term when IL-6 was 2.2

times-higher than its mid-gestation level, does support a threshold effect of IL-6. Unlike IL-6, maternal serum leptin remained unrelated to serum hepcidin at term, which argues against a major role of leptin in modulating hepcidin production during pregnancy. More studies are needed to examine the impact of inflammatory stimuli on hepcidin in populations with varying degrees of inflammation.

There was no evidence for a negative impact of maternal ppBMI and GWG on neonatal Fe status. Consistent with previous findings in adult pregnant women (13), neonates born to obese adolescents had higher cord Hb and total body Fe than those born to normal weight mothers. This observation may reflect greater birth weight and higher red blood cell mass in infants born to mothers with high ppBMI. Similar to the findings in non-diabetic pregnant women (13), there was no significant relationship between maternal BMI and cord SF in pregnant adolescents. It is unclear why we, and others (13), did not observe an inverse relationship between maternal BMI and cord serum Fe as reported previously in 13 healthy pregnant women (4). More studies that undertake a comprehensive assessment of maternal and neonatal Fe status are needed to clarify the impact of maternal weight on neonatal Fe status in pregnant populations.

In this large cohort of healthy pregnant adolescents, maternal obesity and GWG had a limited impact on maternal and neonatal Fe status. Maternal IL-6 increased from mid-gestation to delivery and was positively associated hepcidin at delivery but not at mid-gestation. Maternal IL-6 had no impact on neonatal hepcidin. Our results suggest that low-grade inflammation during pregnancy does not override the suppressive effect of Fe depletion on hepcidin, supporting the notion that hepcidin expression is driven by the strength of each individual regulator under conditions of opposing stimuli.

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CHAPTER 6

CONCLUSIONS

This doctoral research examined the novel hypothesis that heme Fe is a quantitatively important Fe source during pregnancy. Findings from this study have provided new insight into mechanisms by which heme and non-heme Fe are transported across the duodenum and the placenta, the two gateway tissues for Fe delivery to the mother and her fetus during pregnancy. Novel experimental approaches were used to investigate various aspects of Fe utilization in pregnancy including placental heme Fe transporter expression, intestinal Fe absorption and placental Fe transport, the role of hepcidin in regulating cellular Fe transport, as well as the impact of maternal obesity and inflammation on Fe homeostasis across pregnancy. To address these questions, four research projects were undertaken and discussed in detail in Chapters three through six. The major findings from each project are summarized below followed by suggestions for future research.

The first research project was a stable Fe isotope study in rats designed to compare the impact of on duodenal absorption of heme and non-heme Fe. The use of animals allowed us to explore the cellular mechanisms responsible for reduced duodenal Fe absorption in response to hepcidin up-regulation and to further trace the tissue distribution of Fe derived from the two different Fe sources that were ingested. As expected, Fe overload significantly elevated liver hepcidin expression. Hepcidin more potently down-regulated non-heme absorption than heme Fe absorption in rats. In contrast to the classic inhibitory effect of hepcidin on the basolateral Fe exporter FPN which has been described in macrophages, hepcidin had no impact of duodenal FPN but was negatively associated with the apical Fe transporter DMT1. This observation is consistent with emerging literature suggesting that apical Fe uptake, not basolateral Fe export, is

the primary target of hepcidin in the duodenum. Another important and novel finding of this study was the differential distribution of absorbed heme and non-heme Fe between erythroid and Fe storage tissues. A significantly greater proportion of absorbed heme Fe was recovered in the spleen compared to that observed for non-heme Fe, suggesting that some heme may be exported into the circulation in a distinct non-heme Fe form (i.e. as intact heme Fe). Finally, we showed that heme Fe absorption was less efficient than non-heme Fe absorption in rats, while the reverse is true in humans. This raises important questions regarding the choice of appropriate animal models for characterization of heme Fe absorption. Although rat studies offer important insights on the mechanistic aspects of heme Fe absorption, species differences must be considered when making inferences from these observations to human Fe physiology.

In the second project, we generated novel data on placental expression of two cellular heme transporters, the heme-Hx receptor LRP1 and the heme/folate transporter PCFT, in relation to maternal and neonatal Fe status. It is believed that the placenta relies primarily on the uptake of non-heme Fe from the maternal circulation to meet placental and fetal Fe demands. However, the abundant expression of several recently identified cellular heme transporters in the human placenta suggests that the placenta may be able to utilize heme Fe sources. In pregnant adolescents at risk for ID, we observed an inverse association between placental LRP1 expression and cord hepcidin, which may reflect a compensatory up-regulation of placental LRP1 when fetal Fe demands are high. While correlations do not imply causality, this observation is consistent with a role of LRP1 in placental Fe transport. In support of this speculation, LRP1 was found to be positively associated with expression of the heme exporter FLVCR1 in the placenta, which may suggest a coordination of placental heme Fe transport. In the second study under this aim, we assessed placental expression of PCFT, the major intestinal

folate transporter that is known to have a low affinity for heme in the enterocyte, in relation to maternal and neonatal folate and Fe status and to explore its relationships to the two established placental folate transporters, FR α and RFC, in the same pregnant adolescent cohort. Unlike FR α and RFC, which were strongly correlated with cord folate concentrations, PCFT in the placenta was not related to cord serum folate, which is consistent with its undetectable folate transport activity at neutral pH *in vitro*. Together these findings argue against a major role of PCFT in placental folate delivery to the fetus. In addition, we found a positive association between PCFT and cord Fe status. These data may suggest a greater involvement of PCFT in placental heme Fe utilization compared with folate transport. These unique findings regarding placental LRP1 and PCFT provide initial evidence for a possible role of these transporters in placental Fe transport. Further mechanistic studies are needed to elucidate the exact functions of these transporters in placental heme utilization and to delineate the molecular mechanisms involved in the apical uptake, intracellular trafficking, and catabolic degradation of heme in the placenta. Studies are underway in our lab to assess all known heme and non-heme Fe transporters in the human placenta to identify the best predictors of cord Fe status and to examine the relative difference in heme and non-heme transporters in relation to maternal and neonatal Fe status and hepcidin concentrations.

The third research project utilized stable Fe isotope techniques to intrinsically label maternal Hb early in gestation and assess the relative contribution of the maternal RBC pool to neonatal RBC enrichment at birth. Determinants on endogenous Fe transfer to the fetus were compared and contrasted to transfer of dietary non-heme Fe to the fetus using a second stable Fe isotope administered late in the third trimester of pregnancy. This study tested the hypothesis that maternal Fe in the circulating RBC pool is a major source of fetal Fe and examined the

determinants of placental transfer of these two Fe sources. Findings from this study will provide valuable insight on the role of the maternal RBC Fe mass early in gestation and its ability to provide Fe in support of fetal Fe demands.

My final research project was a secondary data analysis that examined possible relationships between maternal obesity and inflammation with maternal and neonatal Fe status in a cohort of 230 pregnant adolescents with a high prevalence of obesity and excessive gestational weight gain. Many epidemiological studies in non-pregnant populations have observed an association between obesity and ID and this association is thought to be mediated in part by inflammation-induced increases in hepcidin which inhibits Fe absorption and Fe mobilization from Fe stores. It is unclear whether a similar relationship between obesity and Fe status occurs during pregnancy and if maternal inflammation affects hepcidin and Fe homeostasis in the mother and her neonate at birth. In the 230 healthy pregnant adolescents studied, pre-pregnancy obesity and excessive gestational weight gain did not negatively impact neonatal Fe status. In contrast, maternal obesity was associated with a significantly higher neonatal Hb concentration at birth. Contrary to the potent stimulatory effect of the pro-inflammatory cytokine IL-6 on hepcidin observed *in vitro*, maternal IL-6 was not related to hepcidin during pregnancy but this association became evident at delivery likely due to delivery mediated changes in inflammation. Our data provide little evidence for a detrimental effect of maternal Class 1 and 2 obesity or excessive gestational weight gain on Fe status in the offspring at birth and suggest that the low-grade inflammation observed during pregnancy in this age group did not override the impact of low Fe stores on the regulation of maternal hepcidin. The differential relationships between IL-6 and hepcidin during pregnancy and at delivery are consistent with the growing literature indicating a threshold effect of IL-6 on hepcidin. More studies are needed to examine the impact

of inflammatory cytokines on hepcidin in populations with varying degrees of inflammation and ID/Fe sufficiency.

Fe homeostasis during pregnancy is maintained through the coordinated regulation of different organ systems to ensure adequate Fe supply for both the mother and her fetus. This doctoral research addressed various important aspects of this complex system as illustrated in **Figure 6.1.** that include: 1) duodenal absorption of dietary heme and non-heme Fe and the role of hepcidin in regulating Fe absorption, 2) placental heme Fe transporters and their relationships to neonatal Fe status, 3) placental transfer of maternal Fe derived endogenously from Hb stores and exogenously from dietary Fe intake, and 4) the impact of maternal obesity and inflammation on maternal and neonatal hepcidin and Fe status.

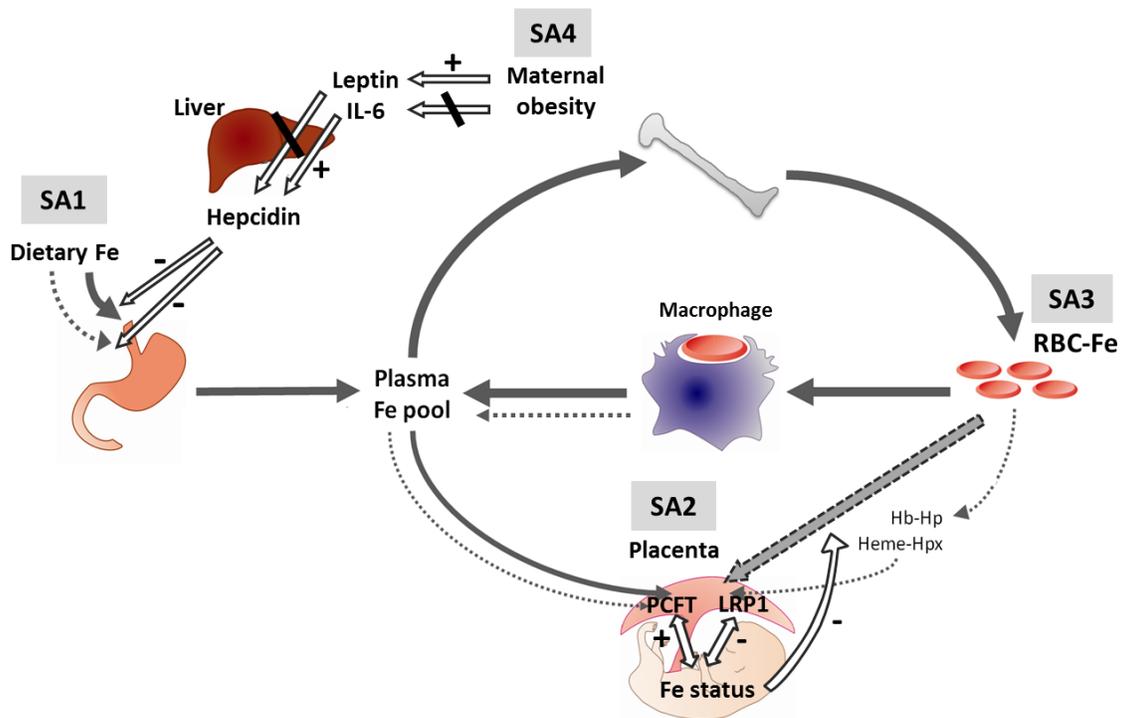


Figure 6.1. Schematic of heme and non-heme Fe flux among different organ systems during pregnancy with the placement of the four specific aims of this research. Solid black lines represent non-heme Fe flux and the dotted black lines represent heme Fe flux. The white arrows represent hypotheses tested in this research. The directionality of significant relationships are indicated by “+” and “-” signs. Arrows crossed with a black line indicate null relationships.

Results from this research underscore the need for additional mechanistic research to: 1) elucidate the molecular mechanisms responsible for the differential effect of hepcidin on heme and non-heme Fe absorption, 2) characterize heme transport activity and cellular locations of the heme Fe transporters in the placenta and identify heme Fe sources in the maternal circulation that are available for placental uptake, 3) investigate the magnitude of intravascular RBC destruction during pregnancy and examine the extent to which placental macrophages participate in RBC catabolism and Hb recycling, and 4) identify additional factors affecting hepcidin production during pregnancy as serum ferritin and inflammation together only account for 20% of the variation in maternal hepcidin at delivery. Polymorphisms in genes involved in Fe homeostasis such as HFE may be major contributors to the large variability in hepcidin and Fe status among individuals. Similar to Fe status and hepcidin, Fe absorption also vary greatly among individuals and hepcidin and Fe stores only capture 30% of the variation in Fe absorption. To explore possible genetic determinants of Fe status and Fe absorption, I participated in a collaborative project in Dr. Zhenglong Gu's laboratory that explored the possible impact of HFE polymorphisms on Fe status and Fe absorption. Dr. Gu's group has previously identified a HFE haplotype that appeared to be under positive selection in East Asia and the subsequent isotope study in young Asian females found greater Fe absorption in the homozygous carriers of this haplotype compared to individuals of the control genotype with similar Fe status, supporting a role of genetic variation in determining Fe absorption and implicating natural selection of efficient Fe utilization as a driving force of these genetic changes. It is also highly likely that gene-gene and gene-nutrient interactions also play a role in regulation of hepcidin production and Fe status. The rapid progress in the understanding of how hepcidin orchestrates whole body Fe metabolism and how hepcidin levels can be altered by peptide-based agonists and antagonists

promises new hepcidin-targeted therapies for common Fe disorders including Fe-restricted anemia in inflammatory diseases and the Fe overload seen in diseases such as hemochromatosis and beta-thalassemia. In addition to systemic Fe homeostasis and transport, emerging data have brought attention to intracellular Fe metabolism and suggested a central role of the mitochondrion in modulating cellular Fe uptake and intracellular Fe trafficking in response to Fe demands of other organelles. Impaired mitochondrial Fe homeostasis has been implicated in the pathophysiology of neurodegenerative diseases, cardiomyopathy, and RBC disorders, which strongly supports the mitochondrial-origin-of-disease hypothesis and opens the possibility of new treatment targets. Knowledge on mitochondrial Fe-processing pathways and the communication between intracellular organelles will add a new dimension to the understanding of Fe metabolism and revolutionize the investigation of Fe-related diseases.

From a public health perspective, this research supports the *Dietary Guidelines for Americans 2010* recommendation for pregnant women to include more heme Fe sources in their diet as heme Fe absorption is less impacted by Fe status and hepcidin. More studies are needed to evaluate the effectiveness, cost, and possible side-effects of heme-based Fe supplementation in pregnancy. In addition, we revealed the previously unappreciated role of maternal RBC mass as an Fe source for the fetus and emphasizes the need to establish an adequate RBC mass prior to pregnancy in support of an adequate fetal Fe endowment at birth.