DETERMINANTS OF MATERNAL AND NEONATAL IRON HOMEOSTASIS IN WOMEN CARRYING MULTIPLE FETUSES AND THEIR NEONATES

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DETERMINANTS OF MATERNAL AND NEONATAL IRON HOMEOSTASIS IN WOMEN CARRYING MULTIPLE FETUSES AND THEIR NEONATES

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Both pregnancy and carrying multiple fetuses increase the iron (Fe) demands across gestation. Adequate Fe stores during pregnancy are essential to support maternal physiological changes and fetal growth. However, currently there are few data available on Fe homeostasis in this high-risk obstetric and neonatal population. This research aimed to provide normative data on maternal and neonatal anemia and Fe status in the context of multiple gestations and identify significant determinants of maternal or neonatal anemia.

Maternal Fe status, inflammatory status, and folate and vitamin B-12 status were monitored across pregnancy (~24 wks, n=73) and at delivery (~35 wks, n=61) in 83 women (aged 20-46 y) carrying multiple fetuses. Neonatal umbilical cord blood samples and placental tissue were obtained from 183 neonates at delivery (25-38 weeks of gestation) to assess neonatal Fe status, inflammation status, and folate and vitamin B-12 status.

A high prevalence of maternal anemia and iron deficiency (ID) was evident in this group of women carrying multiple fetuses. The prevalence of maternal tissue ID (as measured by serum transferrin receptor) increased significantly from pregnancy to delivery. Maternal erythropoietin (EPO) at

either mid-gestation or at delivery was found to be the most sensitive predictor of maternal anemia at delivery. These results suggest that additional screening and Fe supplementation may be warranted in women carrying multiple fetuses.

In this study population, 14% of neonates were anemic at birth. Neonates born to women who were obese prior to pregnancy or smoked cigarettes during pregnancy had significantly greater odds of having anemia at birth, and screening for Fe status in these neonates may be warranted. At this time there are no specific guidelines for Fe status screening in this population and neonates are highlighted for additional screening only if they are born prematurely.

Hepcidin, a systemic regulator of Fe homeostasis, was detectable in umbilical cord blood from nearly all neonates born between 25-38 weeks of gestation in this study. At present, it is not clear when the fetus begins to produce hepcidin, these data add to the normative information available on this topic. In addition, cord hepcidin may regulate cord Fe status independently from maternal hepcidin, as significant associations were only found between cord hepcidin and cord Fe status. Cord hepcidin had a greater intrauterine variance than interuterine variance and significantly impacted the intrauterine variances in cord Hb, serum ferritin, soluble transferrin receptor and EPO, suggesting that cord hepcidin plays an important role in regulation of fetal Fe homeostasis.

BIOGRAPHICAL SKETCH

Yuan Ru was born in Hangzhou, China to Rongchang Ru and Bei Li. She is the only child in the family. From 2008-2012, she attended The State University of New York College in Plattsburgh, NY and obtained her undergraduate degree. There, she majored in Nutrition and minored in Chemistry and Personal Training. After graduation, she decided that research was where she wanted to make a career. In the fall of 2012, she began her doctoral research at Cornell University, NY in the Department of Nutritional Sciences. There, Yuan joined Dr. Kimberly O. O'Brien's research group. While working on her doctoral project, Yuan started her Cornell Dietetic Internship in the summer of 2016. She plans to become a Registered Dietitian in 2017. Yuan's dissertation research was focused on iron homeostasis in women carrying multiple fetuses and their neonates.

DEDICATION

This research is dedicated to the women who participated in my studies, and their infants. Although we never met in person, I will be forever grateful that they allowed me to work with them and share such an important moment in their lives.

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LIST OF ABBREVIATIONS

ACOG	The American Congress of Obstetricians and Gynecologists
APP	American Academy of Pediatrics
ART	Assisted Reproductive Technology
BMI	Body mass index
CDC	Center for Disease Control and Prevention
CRP	C-reactive protein
DMT-1	Divalent Metal Transporter 1
EPO	Erythropoietin
FLVCR	Feline Leukemia Virus Subgroup C Receptor
FPN	Ferroportin
GWG	Gestational Weight Gain
Hct	Hematocrit
Hb	Hemoglobin
IOM	Institute of Medicine
IUGR	Intrauterine growth restriction
ID	Iron Deficiency
IDA	Iron Deficiency Anemia
IL-6	Interleukin-6
Fe	Iron
LBW	Low Birth Weight
NHANES	National Health and Nutrition Examination Survey
RBCs	Red Blood Cells

PTB	Preterm Birth
RDA	Recommended Dietary Allowance
SF	Serum Ferritin
TBI	Total Body Iron
TfR	Transferrin Receptor
USPSTF	The U.S. Preventive Services Task Force
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Specific Aims

Iron (Fe) demands increase dramatically during pregnancy to support maternal plasma volume expansion and to meet the Fe needs of fetal and placental growth. Maternal iron deficiency (ID) and anemia have been increasingly linked to a variety of maternal and neonatal health outcomes. Preterm birth (PTB) and low birth weight (LBW) are common adverse birth outcomes associated with maternal ID and anemia (1). Women carrying multiple fetuses may have higher Fe needs than women carrying singletons, however no specific recommendations for prenatal Fe supplementation are available for this high-risk obstetric group. This merits attention, given that multiple births currently comprise 3.5% of all U.S. births (2) and this group is known to have a significantly higher prevalence of PTB and LBW (3), two adverse birth outcomes that are known to be increased in anemic women.

Early brain development is susceptible to ID *in utero* or depleted Fe stores at birth, as Fe plays an important role in early brain growth and function (4-8). Evidence has suggested that neonatal Fe status may be compromised as a consequence of maternal ID (9-11). Despite the fact that multiple birth neonates are likely to be higher risk of ID and anemia than singleton neonates, few studies have examined the prevalence of anemia in multiple birth neonates. The latest American Academy of Pediatrics (APP) Fe recommendation did not mention multiple birth neonates as a group at higher

risk of ID and anemia (12). At present there are no recommendations for increased Fe status screening in this group.

The placenta is a key mediator of fetal Fe transport. Multiple birth neonates either share a placenta or have their own discrete placenta. At this time, the degree of variability in neonatal Fe status between siblings has not been examined. Hepcidin has been identified as a master regulatory hormone of Fe homeostasis. It inhibits Fe efflux from the cell by internalizing ferroportin (FPN) to trap Fe within the cells that express FPN and by down-regulating the expression of cellular Fe import protein divalent metal transporter 1 (DMT-1) (13-15). A previous study has shown that the fetus starts to produce hepcidin early in the gestation (16). Two recent studies have found that cord hepcidin, but not maternal hepcidin, is significantly associated with neonatal Fe status at birth (17, 18). Little is currently known about hepcidin production in the developing human fetus or the degree to which intrauterine variability in neonatal Fe status is regulated by fetally derived hepcidin.

In order to address the knowledge gaps in these fields, the goals of this research were fourfold: (a) to characterize longitudinal changes in maternal Fe status across pregnancy and at delivery in a group of women carrying multiple fetuses and to identify significant determinants or biomarkers that are most predictive of maternal anemia and ID at delivery; (b) to characterize neonatal anemia and Fe status at birth in a group of multiple birth neonates and to identify significant determinants or biomarkers that are most predictive of neonatal anemia at birth; (c) to evaluate inter- and intrauterine variances in

neonatal Fe status, vitamin B-12 and folate status, and inflammatory status, and (d) to assess the relative impact of neonatal and maternal hepcidin on neonatal Fe homeostasis. The data that were obtained: (a) establish a basis for subsequent Fe screening and nutritional recommendations for women carrying multiple fetuses and their neonates; b) provide a unique approach to identify factors that contribute to placental nutrient transport and partitioning across gestation; and (c) help to achieve the long-term goals of Healthy People 2020, which aim to reduce ID among pregnant women and LBW newborns.

The specific aims and hypotheses were:

Aim I: To characterize longitudinal changes in maternal Fe status across pregnancy and to explore the relationships between Fe status indicators and markers of inflammation.

Hypothesis: *Women carrying multiples will have a higher prevalence of anemia and ID than those carrying singletons. Current standard prenatal Fe supplementation (27 mg of Fe/day) will not be sufficient to maintain maternal Fe status in women carrying multiples.*

Aim II: To characterize neonatal anemia and Fe status at birth and to identify significant maternal determinants or biomarkers that are most predictive of neonatal anemia at birth in newborns born to women carrying multiple fetuses.

Hypothesis: *Neonates born to women carrying multiple fetuses will be at higher risk of anemia at delivery compared to those born to women carrying singletons. Preterm neonates will have higher risk of anemia and depleted Fe stores at birth compared to term neonates. Upregulation of placental Fe transfer will not be sufficient to fully endow the neonate with Fe in the face of maternal anemia and/or insufficient Fe status.*

Aim III: To evaluate inter- and intrauterine variances in neonatal Fe status, vitamin B-12 and folate status, and inflammatory status and to assess the relative impact of neonatal or maternal hepcidin on neonatal Fe homeostasis at birth.

Hypothesis: *Neonatal Fe status will differ between siblings at birth and the intrauterine variance will be driven by cord hepcidin concentrations.*

1.2 Background and Significance

1.2.1 Multiple Births: Prevalence and Pregnancy Outcomes

According to the National Vital Statistics Report, a total of 133,155 twins, 3871 triplets, and 228 quadruplets were born in the United States in 2015. The twin birth rate was 33.5 per 1,000 live births, and the triplet or higher-order birth rate was 103.6 per 100,000 live births (19). There are multiple factors that have led to this rapid increase in multiple births. Advancing maternal childbearing age is a principal cause. The chance of having multiple fetuses doubles as a woman ages. The twin birth rate is 22.4 per 1,000 live births in women aged 20–24 years old, but the rate increases to 51.3 per 1000 live births in women aged 40–44 years old (20). Fertility treatments are another major cause of multiple births. Without fertility interventions, the twin birth rate is 1 per 250 live births, the triplet birth rate is 1 per 10,000 live births, and the quadruplet birth rate is 1 per 700,000 live births (21). Ovulation induction (OI), superovulation (SO), and in-vitro fertilization (IVF) are three common infertility therapies. Ovulation induction and SO are injectable fertility drugs, which stimulate the ovary to produce either a single or multiple oocytes in women with oligo- or anovulation. In-vitro fertilization (IVF) therapies with or without intracytoplasmic sperm injection (ICSI) are more complicated and expensive interventions. Multiple embryos are transferred into the uterus after in-vitro fertilization (22). In the United States, 1% of neonates are conceived with ARTs, accounting for 18% of all multiple births (23).

Women carrying multiple fetuses are at increased risks of adverse birth outcomes, including PTB, LBW, discordant growth, and neonatal mortality (24-26). Multiple birth neonates alone are responsible for 15% of PTB (< 37 weeks of gestation) and contribute 20% of all LBW infants (birth weight < 2,500 g) and 19–24% of all very low birth weight infants (VLBW; birth weight <1,500 g) infants born in the United States (27). It has been well documented that twins who are born after 36 weeks of gestation with birth weights greater than 2,850 g have a higher chance of survival (28-30), however, in most cases twins are delivered at a mean gestational age of 35–36 weeks with birth weights averaging between 2,300–2,600 g (3). Neonates who are born prematurely with LBW have increased risks of both neonatal mortality and morbidity. Multiple birth neonates have a greater than five-fold increase in perinatal mortality and morbidity compared to singleton neonates (31). Discordant growth is defined as a ≥ 15% difference in birth weight between siblings. This is associated with various adverse birth outcomes including intrauterine growth restriction (IUGR), PTB, and stillbirth. Approximately 16% of multiple birth siblings have birth weights that differ by more than 20%, and 5% have birth weights that differ by more than 30% (32).

Interestingly, emerging data have found that while twins have higher rates of LBW than singletons born at the same gestational age, preterm twins have lower gestational age-specific mortality rates than their singleton peers, except for those who are born extremely preterm (< 28 weeks of gestation) (33, 34). This may suggest that twins are programmed to be born prematurely due

to limited space in the uterus. The uterine environment of women carrying multiple fetuses reaches its full capacity at 30–32 weeks of gestation, as at least 80% of multiple fetuses have a combined fetal weight greater than a macrosomic singleton (>4,500 g) at 40 weeks (35). Moreover, carrying multiple fetuses increases the tension on the uterine wall, which causes myometrial stretch that may potentially lead to uterine contractions and consequent preterm labor (36, 37). In addition, the lungs of preterm twins usually undergo an early in-utero maturation after 31 weeks of gestation (38). The early development of lung function may explain why preterm twins have lower mortality rates than preterm singletons. However, the overall perinatal mortality rate of twins is still higher than singletons due to twins' much higher incidence of PTB.

The type of multiples also greatly impacts birth outcomes. In multiple births, zygosity refers to the type of conception and is used to determine whether a set of multiple birth siblings is genetically identical or not. Monozygosity originates from one fertilized egg that splits during the first 2 weeks after conception. Monozygotic siblings are genetically identical and thus have the same gender. In contrast, di-, tri-, or quadrazygosity embryos originate from more than one fertilized egg. Di-, tri-, or quadrazygotic siblings can be born with either the same gender or different genders. Genetically, they are no more alike than any of their siblings (39). There are also multiple possible combinations of zygosity in higher-order multiple births. For instance,

a set of quadruplets can be either four quadrazygotic neonates or a combination of two monozygotic and two dizygotic neonates (39, 40).

Chorionicity refers to the number of placental disks, and amnionicity refers to the number of amniotic sacs for each set of multiples **(see Figure 1.1)**. In a twin pregnancy, if a fertilized egg splits during the first 3 days after fertilization, the fetuses can have either a discrete or fused placenta (dichorionic). If a fertilized egg splits from 4–8 days following fertilization, the fetuses will share a placenta (monochorionic) and each fetus will have their own discrete amniotic sac (diamnionicity). However, if embryonic division occurs between 8 and 13 days after fertilization, it will result in a set of monochorionic monoamniotic twins (MoMo); these twins will share both a placenta and an amniotic sac. Approximately one-third of monozygotic twin pregnancies are monochorionic. While MoMo twins only accounts for only 1% of monochorionic twins, they are at high risk of cord entanglement, which contributes to the increased risk of neonatal mortality. As the two umbilical cords intertwine, blood flow to one or both fetuses may be constricted (40).

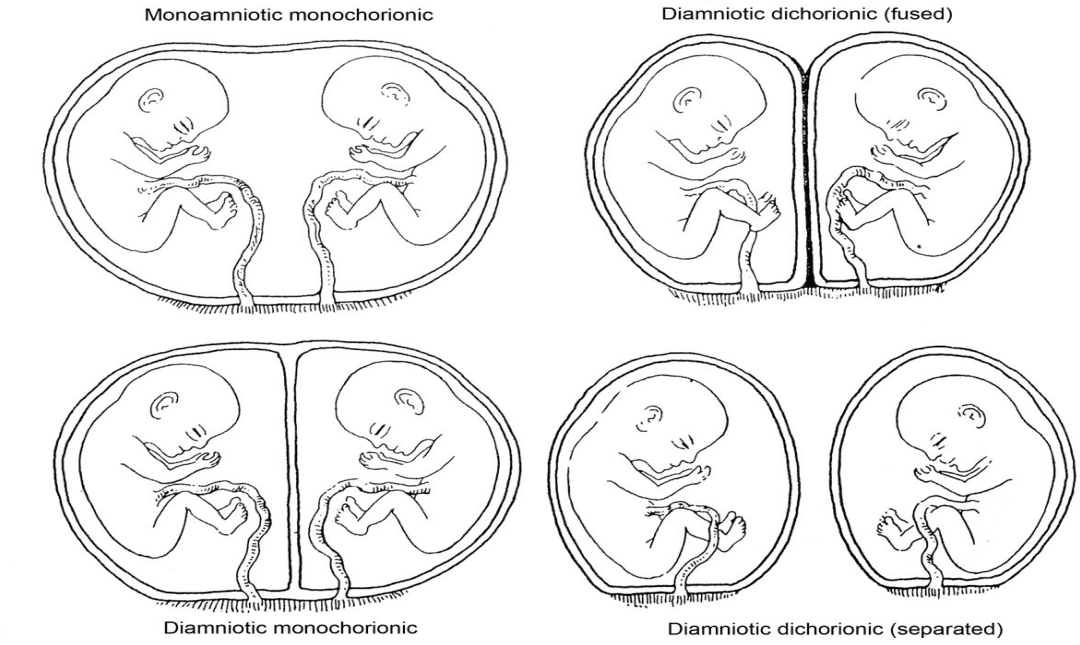


Figure 1.1 Chorionicity and Amnionicity (41)

Monochorionic twins have a three- to four-fold greater risk of perinatal morbidity and mortality compared to dichorionic twins (40). Complications associated with monochorionicity largely stem from the shared placenta and thus the shared vascular supply (39). Competition for nutrients may lead to Twin-Twin Transfusion Syndrome (TTTS), a condition that causes disproportional blood flow between one fetus (the donor) and the other fetus (the recipient). Twin-Twin Transfusion Syndrome is evident in 10–15% of monochorionic twin pregnancies. Several lines of evidence have indicated that unidirectional arterial-venous placental anastomoses are highly associated with TTTS, whereas bidirectional arterial-arterial placental anastomoses are less likely to have TTTS (42, 43). Interestingly, fewer incidences of TTTS have

been reported in MoMo twins when compared to monochorionic diamnionic (MoDi) twins because most MoMo twins have arterial-arterial anastomoses (44).

Lastly, maternal gestational weight gain (GWG) and nutrition status during pregnancy are crucial in relation to neonatal birth outcomes. The 2009 Institute of Medicine (IOM) guidelines set weight gain criteria based on maternal pre-pregnancy BMI (ppBMI) for women carrying twins who deliver at term **(see Table 1.1)** (45). These guidelines aim to optimize birth outcomes for both the mother and her neonates. However, there is a lack of GWG guidelines for underweight women carrying twins and women carrying higher-order multiples due to insufficient data. Women carrying multiple fetuses are likely to gain greater gestational weight compared to women carrying singletons. Excessive GWG is associated with adverse birth outcomes, as the fetus is exposed to high concentrations of lipids. Moreover, inflammatory cytokines derived from adipose tissue may stimulate systemic inflammation (46). However, inadequate GWG is also undesired as it is associated with decreased birth weight (47).

Deficits in essential nutrients can impair fetal growth and development and increase the risk of adverse birth outcomes. The daily recommended caloric intake for a normal-weight woman carrying twins is 40–45 Kcal/kg/day. Demands of specific nutrients, such as Fe, folate, and Ca are also elevated in women carrying multiple fetuses (27). Iron in particular merits attention, given its role in supporting maternal plasma expansion and fetal growth. Iron

deficiency and anemia increase the risk of PTB and LBW. Adequate maternal Fe stores during pregnancy are crucial to prevent to endow the neonatal fully with Fe at birth.

Table 1.1. Institute of Medicine Weight Gain Recommendations for Pregnancy (48)

Pre-pregnancy Weight Category	Body Mass Index	Recommended Range of Total Weight (kg)
Normal Weight	18.5-24.9	16.8-24.5
Overweight	25-29.9	14.1-22.7
Obese (includes all classes)	30 and greater	11.3-19.1

1.2.2 Iron Requirements during Pregnancy

Iron is an essential nutrient for pregnant women for a number of reasons, including: (a) to sustain an increase in maternal plasma volume expansion, (b) to meet the needs of placental development and fetal growth, and (c) to help accommodate the blood loss that occurs during labor (49-51). Approximately 1,240 mg of Fe is required to meet Fe needs for a normal singleton pregnancy, of which about 270 mg is deposited in the fetus (52, 53). In order to accommodate this high Fe demand, a minimum of 500 mg of stored Fe is required by a woman before entering pregnancy. However, 40% of women in the United States have been reported to have no Fe stores at the

time of conception (54). The Recommended Dietary Allowance (RDA) for Fe for nonpregnant women is 18 mg/day; this number increases to 27 mg/day for pregnant women (55). Both the IOM and the Centers for Disease Control and Prevention (CDC) recommend Fe supplementation (27-30 mg/day of Fe) for pregnant women (56, 57) because of insufficient dietary Fe intake as evidenced by the low median dietary Fe intake (15 mg/day of Fe) during pregnancy (57). The U.S. Preventive Services Task Force (USPSTF), however, reports that the beneficial effects of Fe supplementation on maternal and neonatal health outcomes in the Unites States are still inconsistent (58).

Iron demands vary from one trimester to the next during pregnancy, increasing nearly ten-fold from 0.8 mg/day in the first trimester to 7.5 mg/day in the third trimester **(see Figure 1.2)** (52). During the first trimester, Fe requirements decrease due to the cessation of menstruation resulting in a saving of 0.56 mg of Fe per day (59). Obligatory Fe from the gut, skin, and urine are the primary Fe losses during the first trimester (60). During the second trimester, Fe requirements begin to elevate due to hemodynamic changes, by which maternal plasma volume and red blood cell mass expand up to 50% and 35%, respectively (~450 mg of Fe) (1). Maternal ID is likely to develop during the third trimester of pregnancy, as a large amount of Fe is transferred from maternal circulation to the fetus. In support of this statement, data from the National Health and Nutrition Examination Survey (NHANES) have found that the prevalence of ID in pregnant women carrying singletons (*n* = 1171) increases from 7% in the first trimester to 30% in the third trimester

(61). The additional Fe losses that occur at delivery also need to be taken into account, as a total of 240 mg of Fe is lost from maternal blood, placenta, and umbilical cord losses (62). Since the current Fe requirements is estimated based on data obtained from women carrying singletons, it is unclear the Fe demands for women carrying multiple fetuses. Compared to a women carrying singletons who has approximately 1,570 mL increase in blood volume, a woman carrying multiple fetuses has greater increase in blood volume during pregnancy (1,960 mL for a twin pregnancy) (63), and therefore might have higher Fe demands during pregnancy. This highlights the need for further data on Fe homeostasis in this high-risk obstetric population.

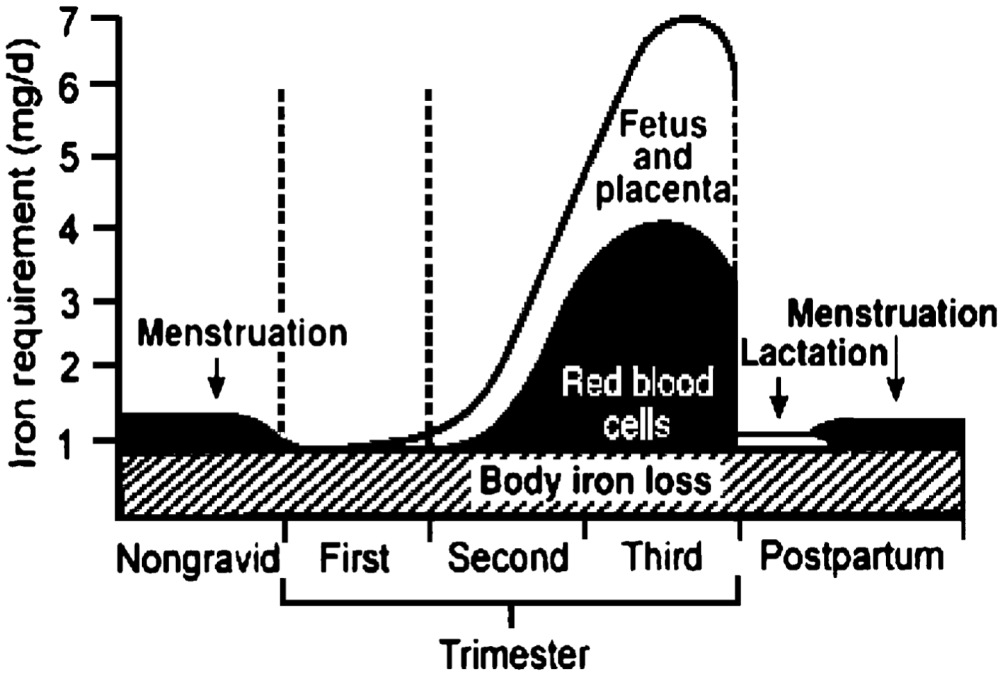


Figure 1.2: Estimation of Daily Fe Requirements during Pregnancy (52)

1.2.3 Iron Metabolism During Pregnancy

The placenta is a unique temporary organ that mediates the transmission of nutrients from maternal circulation to the fetus. Humans have a hemochorial placenta which means that maternal blood comes into direct contact with the fetal chorion (64). Nutrients can therefore be rapidly transferred to the fetus across the two cell layers (syncytiotrophoblast and fetal endothelia cells) in the chorionic villi (64). The syncytiotrophoblast (STB) is the epithelium on the placental villi that interfaces with maternal blood and plays an important role in nutrient exchange. The STB selectively transports essential nutrients to meet the needs of placental and fetal development; meanwhile it secretes fetal waste products back into maternal circulation. During the third trimester of pregnancy, large amounts of Fe are actively transported across the STB against a concentration gradient to meet increased demands for fetal Fe stores. The rate of placental Fe transport sets the stage for the neonatal Fe endowment at birth and helps to prevent development of ID during infancy.

During pregnancy, the fetus obtains Fe from the maternal diet and catabolism of senescent red blood cells (RBCs). In pregnant women, approximately 3-4 mg of Fe is absorbed from the maternal diet, and ~20 mg of Fe is released daily into the circulation from RBC catabolism. Using stable Fe isotopes, human data published from our laboratory supported a unidirectional Fe transfer from maternal to fetal circulation (65, 66). The hypothesized mechanism of placental Fe transfer is depicted in **Figure 1.3** (67). With this model, ferric Fe (Fe^{3+}) in the maternal blood is first bound to maternal

transferrin (Tf), and this complex binds to TfR at a greater affinity when PH is 7.4 (68). Transferrin receptor protein is expressed apically on the STB membrane and mediates the complex to be endocytosed. Iron is then dissociated from the Tf/TfR complex and is reduced to its ferrous state by Steap 1, 2, 3, and 4 (67). Steap 1, 2, 3, and 4 are members of the Steap family of proteins (six-transmembrane epithelial antigen of the prostate) and play important roles as metalloreductases for Fe and copper (69). Both Steap 3 and 4 are highly expressed in the placenta and exhibit the redundant function to transfer Fe across the placenta to the fetus (69).

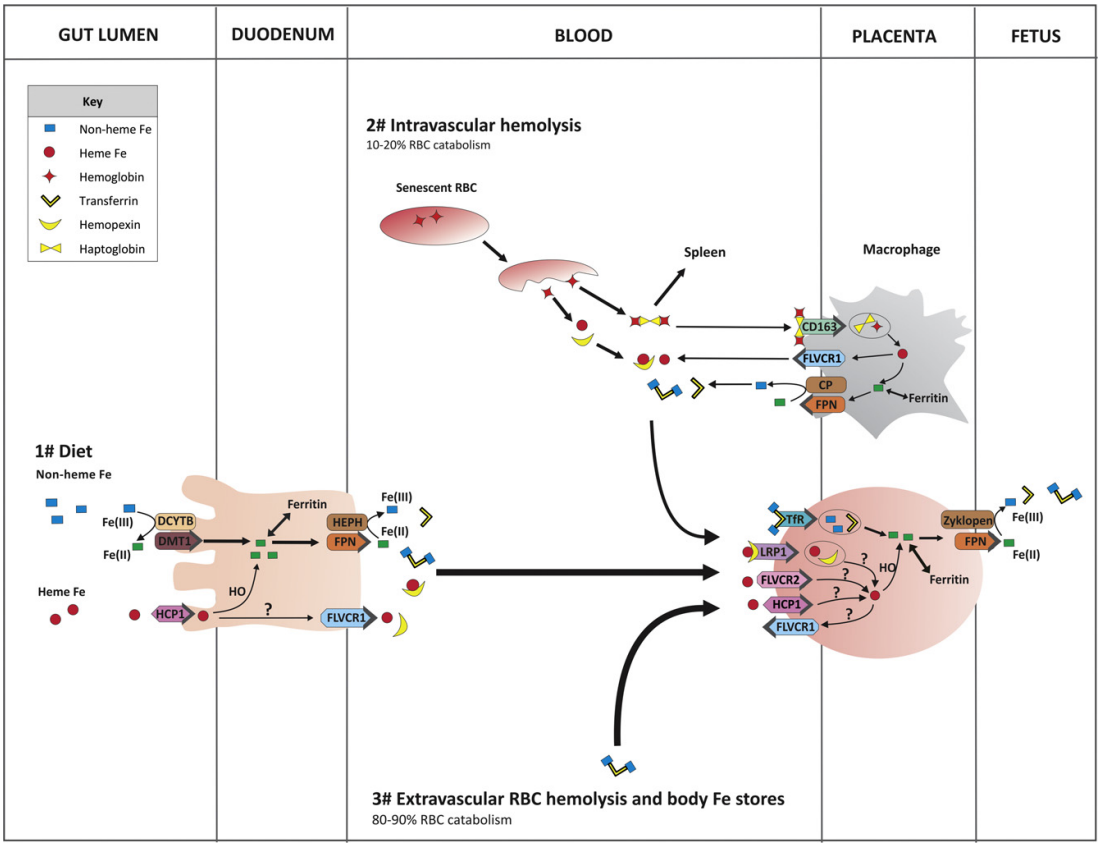


Figure 1.3. Iron Transport Across the Placenta (70)

Once ferric Fe (Fe^{3+}) is reduced to ferrous Fe (Fe^{2+}) by Steap 3 and 4 in the endosome, ferrous Fe is transported by a cellular importer protein, DMT-1, across the endosmal membrane into the cytoplasm (71). Although DMT-1 is an important import protein for nonheme Fe uptake and is richly expressed in the human placenta, the essentiality of DMT-1 for placental Fe transfer has been controversial (72). Animal studies have shown that DMT-1 is dispensable for placental Fe transport. Total body Fe content of DMT-1 globally knockout mice at birth was not significantly different from total body Fe content in wild type, indicating that DMT-1 is not essential for maternal-fetal Fe transfer in the

mouse (73, 74). Alternative or complementary pathways (i.e., Zip 8 and Zip 14) must exist in addition to DMT-1 for placental Fe transport in this animal model (75, 76). Zip 8 (SLC39A8) is a transmembrane protein that belongs to the Zip-family of metal-ion transporters. It is closely related to Zip 14, another Zip-family protein that transports Fe and mediates uptake of non-transferrin bounding Fe (NTBI) (77, 78). Both Zip 8 and Zip 14 are abundantly expressed in the human placenta (76, 79)

Once in the cytoplasm, ferrous Fe is exported from the basolateral side of the placental STB into fetal circulation. Ferroportin (FPN) is the only known nonheme Fe exporter. Knockout FPN mice exhibit embryonic lethality due to severe iron depletion (80). As noted, there is no known recycling of nonheme Fe from fetal to maternal circulation. Once Fe is in the cytoplasm, it is either transferred to the fetus or stored as ferritin. A ferroxidase called zyklopen is thought to replace the enterocyte protein—hephaestin and ceruloplasmin—in the placenta to re-oxidize ferrous Fe to ferric Fe, so that the Fe can be loaded on fetal Tf (81).

Unlike enterocytes that respond only to only maternal Fe status, the placenta regulates Fe transport in response to both maternal and fetal Fe status. Placental nonheme Fe transfer is upregulated in response to maternal ID, suggesting that placental Fe transfer can adapt to maternal Fe supply (82, 83). Using an ID rat model, compensatory changes were found to occur in placental Fe transport as evidenced by an increase in the activity of copper oxidase and the expression of TfR and DMT-1 in this model (84). Human data

have also found that placental TfR expression was elevated in mothers with depleted body Fe stores (85). The relative importance of fetal Fe status in regulating placental Fe transport is still unclear. In a rat model, a stronger correlation was found between the placental expression of TfR and fetal liver Fe than between TfR and maternal liver Fe, indicating that fetal liver Fe may be a key determinant of placental Fe transfer (72).

1.2.4 Assessment of Maternal and Neonatal Iron Status and Inflammation

Multiple Fe status indicators can be used to assess maternal and neonatal Fe status and to define anemia and iron ID **(see Table 1.2)**. Hemoglobin (Hb) is a protein in the red blood cells that carries oxygen to the tissues, and nearly 80% of the Fe in the body is found in Hb. Therefore, measures of Hb in the blood reflect the amount of the circulating red cell mass, and this measure is commonly used in the clinical setting to define anemia. Similar to Hb, hematocrit (Hct) is also widely used to determine the percentage of red blood cells in the blood.

Table 1.2 Summary of Iron Status Indicators (86)

Iron Status Indicator	Definition	Advantage
Hemoglobin (Hb)	Measure of anemia; Reflects amount of functional Fe in body	Inexpensive; Universally available; Simple to measure
Hematocrit (Hct)	Packed cell volume; Proportional volume of RBCs in whole blood; Reflects amount of functional Fe in body	Same as Hb
Serum Ferritin (SF)	Storage Fe; Reflects total body Fe stores; Acute phase protein	Quantitative; Well standardized
Soluble Transferrin Receptor (sTfR)	Indicator of tissue Fe availability; Reflects balance between cellular Fe requirements and Fe supply	Quantitative; Unaffected by inflammation
Erythropoietin (EPO)	A hormone that increase the rate of RBC production	
Serum Iron	Indicator of the amount of circulating iron that is bound to transferrin	Serum iron is not a sensitive measure of iron deficiency due to daily fluctuations
Hepcidin	Regulatory hormone of Fe homeostasis; Affected by inflammation	Production diminished when iron reserves depleted
Total Body Iron	Maternal TBI: Body iron (mg/kg)=-[log10 (sTfR/SF)-2.8229] ÷0.1207 (87); Neonatal TBI: the equation described by Siddappa *et al* with adjustment for gestational age at birth (88)	Measure of full range of iron status, validated by phlebotomy studies in adult volunteers

As a result of the increase in plasma volume during pregnancy, the Hb concentration cut-offs used to define anemia vary by trimester. To account for the known change in Hb, anemia is defined as a Hb concentration less than 11 g/dL in the first (< 14 weeks of gestation) and third trimester (≥ 28 weeks of gestation), and as a Hb concentration less than 10.5 g/dL in the second trimester (14–27 weeks of gestation) (89). Compared to Caucasian women, African-American women tend to have a shift to the left in the Hb distribution curve (90). The IOM defines anemia in the African-American population as 10.2 g/dL in the first and third trimester, and 9.7 g/dL in the second trimester (91). Women who smoke cigarettes or live at a high altitude tend to have higher Hb and Hct concentrations, and therefore adjusted higher cut-off values of anemia are used in these instances. Many studies have reported a U-shaped relationship between Hb and adverse birth outcomes (92-95), suggesting that the risk of adverse birth outcomes is higher at both ends of the Hb range.

Neonatal anemia is defined as central venous Hb concentrations less than 13 g/dL or capillary Hb concentrations less than 14.5 g/dL (96). Causes of neonatal anemia are complex and varied including blood loss (i.e. hemorrhage or poor feto-maternal transfusion), RBC destruction (i.e. hemolysis or infection), or decreased RBC production (i.e. depleted Fe stores or bone marrow suppression). Various factors including site of blood collection, time of blood collection, position of neonate after delivery, and the timing of umbilical cord clamping at delivery can also affect the interpretation of

neonatal Hb data. Hemoglobin concentrations measured in capillary blood samples are 5-10% higher than those measured in venous blood samples (97). Increased Hb concentrations are also found in the blood that collected immediately after delivery mainly due to placental transfusion (98). After delivery, the position of the neonate also influences Hb concentrations, as neonates who were held below the level of the placenta would continuously receive blood from the placenta (99). The timing of umbilical cord clamping is another factor that affects Hb concentrations in newborns. Three-minute delayed cord clamping has been found to allow for the placental transfusion of 80-100 mL of blood (approximately 40-50 mg/kg of Fe) to the neonate (100). Data reported by the 2012 Cochrane systemic review defined delayed cord clamping as a delay of more than 30 seconds after birth and found the beneficial effect of delayed cord clamping on lowering risk of transfusion for anemia in a cohort of 738 newborns (101). Data reported by the 2013 Cochrane review continued to supported this practice, as they found that delayed cord clamping (>1 min after birth) was associated with higher Hb and SF concentrations in the first 6 months of life in a cohort of 3,911 newborns (102). At present, the American College of Obstetricians and Gynecologists (ACOG) recommends a delay in cord clamping (for at least 30-60 seconds after birth) in both term and preterm neonates to improve birth outcomes (103).

Both Hb and Hct have low sensitivity and specificity to identify ID during pregnancy, largely due to the expansion of plasma volume and red cell mass associated hemodilution in pregnancy. Also, given the interpersonal variation,

the degree of hemodilution varies among individuals. A woman with the same red cell mass may have different measures of Hb and Hct (52, 104). Moreover, Hb and Hct concentrations can be influenced by other nutrients or complications. Folate or vitamin B-12 deficiencies and chronic inflammation can lower Hb concentrations. To determine whether anemia is a consequence of ID, serum ferritin (SF) and soluble transferrin receptor (sTfR) can be utilized to estimate Fe stores and tissue Fe availability, respectively.

Serum ferritin is the main Fe storage protein. It is produced in the liver, and production increases as body Fe supplies increase. In healthy non-pregnant adults, 1 μg/L of SF is equivalent to 8–10 mg of storage Fe; in newborns, 1 μg/L of SF is equivalent to 2.7 mg of stored Fe (105). Depleted Fe stores in pregnant women is defined as a SF concentration less than 12 μg/L (106). This definition has been used in the most recent NHANES study (1999-2006) (107). To date, there is no specific SF cut-point to define neonatal ID at birth. A previous study has found that cord SF concentrations less than 76 μg/L were associated with impaired cognitive development (108). However, emerging evidence indicates that cord SF might not be a good indicator of Fe status in newborns as an unexpected negative association was found between cord SF and cord Hb in neonates born to pregnant adolescents and women carrying multiples (18, 109).

While SF is a good Fe status indicator to assess ID, it also has several limitations that might interfere with its interpretation. First, SF concentrations are impacted by hemodynamic changes as hemodilution during pregnancy

could lower SF concentrations (110). Moreover, SF has low sensitivity to true ID because of its large intrapersonal variation (111). Finally, SF is an acute phase protein, increasing as a consequence of inflammation, and use of this biomarker alone may mask the real ID in the population (112, 113). More information regarding SF and inflammation will be discussed in the following paragraphs.

Transferrin receptors are expressed on the surface of cells to facilitate Fe transport across the cell membrane. Transferrin receptor is up-regulated when tissue Fe is depleted to enhance uptake and transport Fe into the cell (114). When TfR is released into the serum, it can be analyzed as soluble TfR (sTfR). Unlike SF, sTfR concentrations are not influenced by inflammation (115, 116) and have high specificity to tissue ID (117), thus they can be used as a good Fe status indicator to define ID when SF concentrations increase in response to inflammation (116). Tissue ID is defined as sTfR concentrations greater than 8.5 mg/L based on data obtained from quantitative phlebotomy in adults (118). Neonatal sTfR concentrations are impacted by the fetal erythropoietic activity (119). However, at this time no sTfR cut-point value is available to define neonatal tissue ID at birth, as data on umbilical cord blood sTfR are limited.

With both SF and sTfR, total body iron (TBI) in pregnant women can be calculated using the equation TBI (mg/kg) = -[$\log_{10}$ (sTfR x 1000/ferritin)-2.8229]/0.1207] described by Cook et al. (87). A TBI less than 0 mg/kg indicates body ID, as validated in pregnant women (61).

Erythropoietin (EPO) is a hormone that regulates the production of erythrocytes in mammals. During pregnancy, EPO concentrations steadily increase to support the high oxygen demands, reaching a maximum at the end of the third trimester (120). Similarly, in newborns high EPO concentrations stimulate the production of red blood cells by neonatal bone marrow. As serum EPO concentrations respond to both maternal (121, 122) and neonatal ID (32), they can be used as a Fe status indictor. Despite the fact that the efficacy of EPO as a treatment option for anemia in pregnancy is well recognized (123), there has been a lack of attention to its value as an indicator to monitor anemia and Fe status in pregnancy. Data published from our lab have demonstrated that serum EPO is not affected by inflammation and is a significant predictor for maternal Hb concentrations and ID across pregnancy in a group of pregnant adolescents (124).

Hepcidin has been identified as the systemic regulator of Fe homeostasis (125). It is a 25-amino acid polypeptide synthesized in the liver and encoded by the *HAMP gene* (126). It limits Fe absorption by internalizing FPN and suppressing DMT-1 in the enterocyte to trap Fe within the cell (13-15). Hepcidin production is stimulated by body Fe stores or inflammation and is down-regulated by erythropoietin activity or hypoxia (127). Interleukin-6 (IL-6) is the primary inflammatory signal that up-regulates production of hepcidin (128), acting through the promoter binding of the Signal Transducer and Activator of Transcription 3 (STAT 3) (129). Studies of chronic infections and severe inflammatory diseases have shown significantly increased hepcidin

concentrations (130), supporting the role of hepcidin as an acute phase protein. Erythropoiesis suppresses hepcidin production through erythroferrone, a newly identified hormone stimulated by EPO (131, 132). Erythroferrone-deficient mice have elevated hepcidin after hemorrhage (131). Currently, there are multiple methods available to measure hepcidin concentrations. Consequently, reference intervals and detection limits for hepcidin are method-dependent, and it is challenging to make comparisons between hepcidin concentrations measured by different methods (133). There is no standardized quantification method to measure hepcidin and the cost of analysis is generally high.

As mentioned above, SF and hepcidin are acute phase proteins that increase in response to inflammation. Pregnancy *per se* is a pro-inflammatory and anti-inflammatory environment, which varies based on the stage of pregnancy. During the implantation and placentation period in early gestation, the blastocyst breaks through the epithelial lining of the uterus for implantation and damages the endometrial tissue, followed by the trophoblast replacement of endothelium and vascular smooth muscle of maternal blood vessels (134). All of these changes create a pro-inflammatory environment in order to support a successful implantation. During the mid-gestation period, an anti-inflammatory environment occurs, as this is a period of time for rapid fetal growth (135). The late stage of pregnancy returns to a pro-inflammatory environment for parturition (136, 137). Therefore, it is important to include

inflammatory markers when interpreting Fe status indicators to avoid underestimating the prevalence of ID.

Interleukin-6 is the primary inflammatory signal that up-regulates production of hepcidin (128). Previous data have found significantly increased IL-6 concentrations from pregnancy to delivery (138, 139), largely because IL-6 plays an important role in the parturition process (137, 139). C-reactive protein is another inflammatory marker, which is produced by the liver and is induced by IL-6 (140). Some studies have reported no change in CRP concentrations across gestation (141, 142), whereas others have showed increased concentrations as a function of gestational age (143). Both CRP and IL-6 should be measured in order to interpret SF or hepcidin results and account for the degree of pregnancy-associated inflammation. While normative correction factors for inflammation have been recently developed to adjust SF for the nonpregnant population (144, 145), at this time no correction values are yet available to adjust SF or hepcidin for the pregnant population.

1.2.5 Impact of Maternal Iron Deficiency on Neonatal Iron Status

As noted above, maternal ID increases the risk of adverse birth outcomes including PTB and LBW. The mechanism of how maternal ID influences neonatal birth outcomes is still unclear. Hypoxia, oxidative stress, and infections need to be considered. Hypoxia induced by ID can trigger a stress response to release corticotrophin-releasing hormone (CRH) from the placenta and cortisol from the fetus; both hormones are associated with

increased risk of PTB. Additionally, oxidative stress may cause damage to placental development, and consequently contribute to the risk of PTB (146). Since full-term neonates generally accrue more than two-thirds of their total body Fe during the third trimester, being born early in the third trimester reduces the time that the fetus can accrete Fe during this critical growth window. As multiple-birth neonates are more likely to be born prematurely compared to singleton neonates, they are at a higher risk of ID at birth.

It is believed that Fe metabolism during pregnancy favors fetal Fe acquisition even in the face of maternal anemia. However, emerging evidence suggests that fetal Fe needs can be compromised under conditions of maternal ID (147-149). If Fe supply is limited, Fe will be prioritized to erythropoiesis at the cost of brain and heart development (150). Neonatal Fe stores at birth are essential to maintain Fe status across early infancy because the neonatal gut is not fully developed and may not be able to appropriately regulate Fe absorption in response to Fe status until 6–9 months of age (151, 152). Depleted Fe stores at birth have been associated with long-term, irreversible motor-cognitive impairment during infancy (153, 154).

1.2.6 Hepcidin and Its Regulation on Neonatal Iron Homeostasis

Hepcidin is a systemic regulator of Fe homeostasis in adults (155). It regulates Fe status by internalizing the expression of the Fe export protein FPN and suppressing the expression of Fe importer protein DMT-1 (13-15). Hepcidin production can be assessed by examining liver hepcidin mRNA

levels (in animal models) or by measuring serum hepcidin (in human studies) (156). Hepcidin mRNA and protein levels are highly correlated as production of hepcidin is predominantly regulated at the transcriptional level (146).

In human studies, maternal hepcidin concentrations have been positively associated with maternal SF and negatively associated with sTfR across pregnancy (157-159). At delivery, the lowest hepcidin concentrations have been found in women with depleted Fe status (157, 160). Our lab was the first to publish data on the association between serum hepcidin and Fe absorption in humans. We found that serum hepcidin was significantly inversely associated with nonheme Fe absorption in healthy nonpregnant women (161). We also found that hepcidin was inversely associated with placental Fe transfer in healthy pregnant women (162).

The fetus begins to produce hepcidin early in the first trimester as determined by the presence of pro-hepcidin (precursor of hepcidin) in fetal blood obtained at 8–11 weeks of gestation (163). Several human studies examining maternal and cord hepcidin concentrations at mid-gestation and delivery have found significantly lower maternal hepcidin concentrations than cord hepcidin concentrations (157, 160, 164, 165). Low maternal hepcidin concentrations during pregnancy were thought to ensure sufficient maternal Fe absorption and Fe efflux from macrophages and hepatic Fe stores in support of increased fetal Fe demands (157). Several recent studies measuring Fe status indicators in maternal and neonatal dyads have found that cord hepcidin—but not maternal hepcidin—is significantly associated with

neonatal Fe status at birth (17, 18), indicating that fetally-derived hepcidin may independently regulate fetal Fe status. An Fe absorption study using both heme and nonheme stable Fe isotopes by our laboratory found that Fe transfer to the fetus was inversely correlated with maternal hepcidin and positively associated with neonatal hemoglobin (162), indicating that maternal hepcidin may also have some control over fetal Fe homeostasis. However, the relative contribution of maternal versus fetal hepcidin in the regulation of placental Fe transport is not well understood.

1.2.6 Multiple Births Study Design

Subject Recruitment and Eligibility

This research was undertaken at the School of Medicine at the University of Rochester (UofR), in Rochester, New York. The maternal and fetal medicine program at the Strong Memorial Hospital in Rochester is one of the leading centers for high-risk deliveries in Upstate New York. Each year there are approximately 3,000 deliveries occurring at this hospital. A total of 83 women carrying multiples and their neonates (n = 183) were recruited for the study between the period of 2011–2014.

Pregnant women carrying twins, triplets, or quadruplets between the ages of 19–45 years were identified when entering prenatal care. Women were approached by our health project coordinator, who was hired full-time to work on this study. Recruitment posters, flyers, and cards were distributed in obstetric offices that treat women carrying multiples. Eligible women were

those who were otherwise healthy and had no diagnosed or pre-existing medical conditions known to impact Fe homeostasis (i.e., hemoglobinopathies, eating disorders, malabsorption, diseases, steroid use, and substance abuse history). Eligibility was confirmed by our study Co-Investigator, Dr. Eva Pressman, the Henry A. Thiede Professor and Chair of the Department of Obstetrics and Gynecology at UofR. Once eligibility was confirmed, women were invited to participate in this longitudinal study of Fe homeostasis across pregnancy and delivery in the maternal/neonatal dyad. Informed written consent was obtained from all study participants. The Institutional Review Boards (IRB) at Cornell University and the University of Rochester approved this study.

Sample/Data Collection

All participants were followed longitudinally across pregnancy for data collection **(see Table 1.3).** Demographic and health information was self-reported at baseline using a validated health survey questionnaire. With the assistance of the study coordinator, each participant completed an Eating Habit and Beliefs Interview and a Nutrition Questionnaire. Since all participants were prescribed standard prenatal supplements (containing 27 mg of Fe) as a part of their prenatal care, a Prenatal Supplements Questionnaire was administered to assess the type and frequency of prenatal supplement use. Frequency of prenatal supplement use was described as “prescribed frequency” and “actual frequency” and categorized as: every day, 2–5 times a

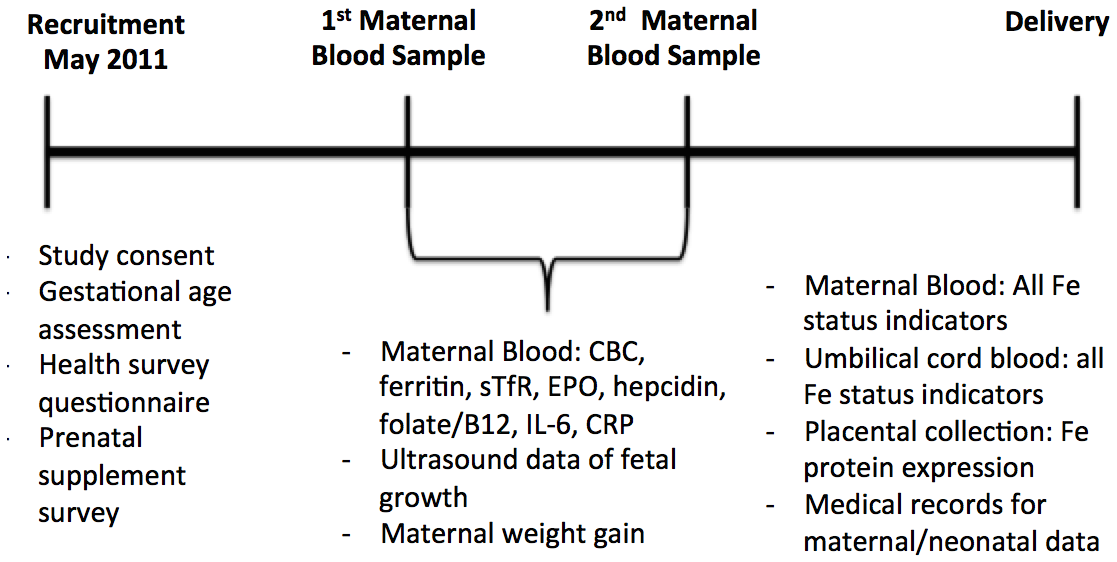
week, once a week, occasionally, very rarely, or never. The study coordinator filled out a validated Baby Data Form after delivery. It included delivery date and time, GA at delivery, neonatal sex, delivery duration, delivery mode, delivery complications, neonatal weight/length/head circumference, and APGAR scores. Other neonatal characteristics including the type of placentation (chorionicity/amnionicity), infection/inflammation, and neonatal complications at birth were abstracted from pathology reports and medical records.

Table 1.3: Summary of Collected Data

Participants Characteristics	Baby Data From	Maternal/Neonatal Fe status Indicators	Placenta
• Age at baseline/delivery • Race/ethnicity • Gestational age at baseline/delivery • Parity • Pre-pregnancy height/weight/BMI • Weight/BMI at delivery • Cigarette, alcohol & drugs use • Blood pressure • Supplement use questionnaire • Type of multiple births • ART use/ donor egg/sperm	• Neonatal weight/length/head circumference • Neonatal gender • Apgar score • Delivery mode/antepartum/ pregnancy induced/ delivery complications	• Complete blood count: Hb, Hct, etc. • Serum iron • Serum transferrin receptor • Total body iron • Serum hepcidin • Erythropoietin • Serum iron • C-reactive protein • Interleukin-6 • Serum folate/B-12	• Weight/length • Width/volume • Inflammation • Surface area • Type of placentation • RNA/protein expression

Maternal non-fasting blood and neonatal umbilical cord blood sample was collected at each study visit and/or at delivery **(see Figure 1.4)**.

Figure 1.4: Study Design



From the maternal blood (15 mL) and umbilical cord blood (20 mL) samples obtained, whole blood was used to assess maternal/neonatal hematological status. Serum was separated on-site and stored at -80^{o}C until it was transported on dry ice to Cornell University, NY for further analysis of Fe status indicators, hepcidin, inflammatory markers, vitamin B-12, and folate. All participants' placentas were collected after delivery and sent to the pathology department at Strong Memorial Hospital to be evaluated by placental pathologists. Medical charts were reviewed to obtain maternal delivery and neonatal birth data.

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CHAPTER 2

IRON DEFICIENCY AND ANEMIA ARE PREVALENT IN WOMEN WITH MULTIPLE GESTATIONS

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2.1. ABSTRACT

Background: Little attention has been placed on the unique iron demands that may exist in women with multiple gestations. This merits attention, as iron deficiency during pregnancy is associated with adverse pregnancy outcomes that are known to be more prevalent among multiple births.

Objective: To characterize longitudinal changes in maternal iron status across pregnancy in a cohort of healthy women with multiple gestations and identify significant determinants of maternal iron deficiency and anemia.

Design: A group of 83 women carrying twins, triplets, and quadruplets (aged 20-46 y) was recruited from 2011-2014. Blood samples obtained during pregnancy (~24 wks, n=73) and/or at delivery (~35 wks, n=61) were used to assess hemoglobin, serum ferritin (SF), soluble transferrin receptor (sTfR), hepcidin, serum iron, erythropoietin, serum folate, vitamin B-12, C-reactive protein and interleukin-6.

Results: The prevalence of tissue iron deficiency (sTfR > 8.5 mg/L) increased significantly from 9.6% during pregnancy to 23% at delivery (P = 0.03). Of women (n=20) with depleted iron stores (SF < 12 μg/L) during pregnancy, 25% (n=5) developed iron deficiency anemia (IDA) at delivery. Women with depleted iron stores during pregnancy also had a 2-fold greater risk of anemia at delivery. Nearly half of women studied (44.6%, n=37/83) were anemic at delivery and 18% (n=11/61) had IDA. Erythropoietin during pregnancy was significantly negatively associated with hemoglobin at delivery. Women with erythropoietin >75th percentile during pregnancy exhibited a 3-fold greater risk

of anemia, suggesting that erythropoietin is a sensitive predictor of anemia at delivery. Inflammation was present at delivery, limiting the utility of ferritin or hepcidin as iron status indicators at delivery.

Conclusion: Iron deficiency and anemia are highly prevalent in women with multiple gestations. Additional screening and iron supplementation may be warranted in this high-risk population, given the known associations between iron deficiency, anemia and adverse maternal/neonatal outcomes.

2.2 INTRODUCTION

In the United States, national survey data indicates that anemia and iron deficiency (ID) are evident in 5.4% and 18% of pregnant women carrying singletons, respectively (1). Anemia and ID during pregnancy have been associated with increased risk of a number of adverse birth outcomes, including preterm birth (PTB), low birth weight (LBW), and low neonatal iron (Fe) stores at birth (2-4). While the optimal amount of storage Fe at birth is unknown, growing data suggest that the developing fetal brain is susceptible to Fe insufficiency in utero and low Fe stores at birth have been associated with motor-cognitive deficits in infants (5-8).

Despite the growing body of research on Fe homeostasis during pregnancy, very little is known about Fe status in women with multiple gestations, a population that may have higher Fe demands during pregnancy. This is of concern as multiple births now comprise 3.5% of all births in the United States (9) and this group alone is responsible for 15% of PTB and

contributes 20% of the LBW infants born in the United States (10).

Current prenatal recommendations advocate use of an iron containing prenatal supplement across gestation, and women with multiple gestations are advised to adhere to the same guidelines as women carrying singletons. It is unknown if the typical Fe content of a standard prenatal supplement (27 mg) is sufficient for all higher risk obstetric groups. To date few data on Fe status and anemia exist in otherwise healthy women with multiple gestations (11, 12) or in women with multiple gestations who experience pregnancy complications (13, 14). The last published American College of Obstetricians and Gynecologists (ACOG) practice bulletin on anemia in pregnancy did not identify women carrying multiple fetuses as an at-risk obstetric population and made no additional Fe intake recommendations for this group (15).

To address this current knowledge gap, the goal of this study was to characterize longitudinal changes in maternal Fe status across pregnancy in a cohort of healthy women with multiple gestations and to identify significant determinants of maternal ID and anemia by exploring relationships between Fe status indicators, hepcidin, and inflammatory indicators across pregnancy and at delivery.

2.3 SUBJECTS AND METHODS

Participants

A cohort of 86 women (aged 20-46 y) carrying twins (n=66), triplets (n=19), or quadruplets (n=1) was recruited to participate in a prospective

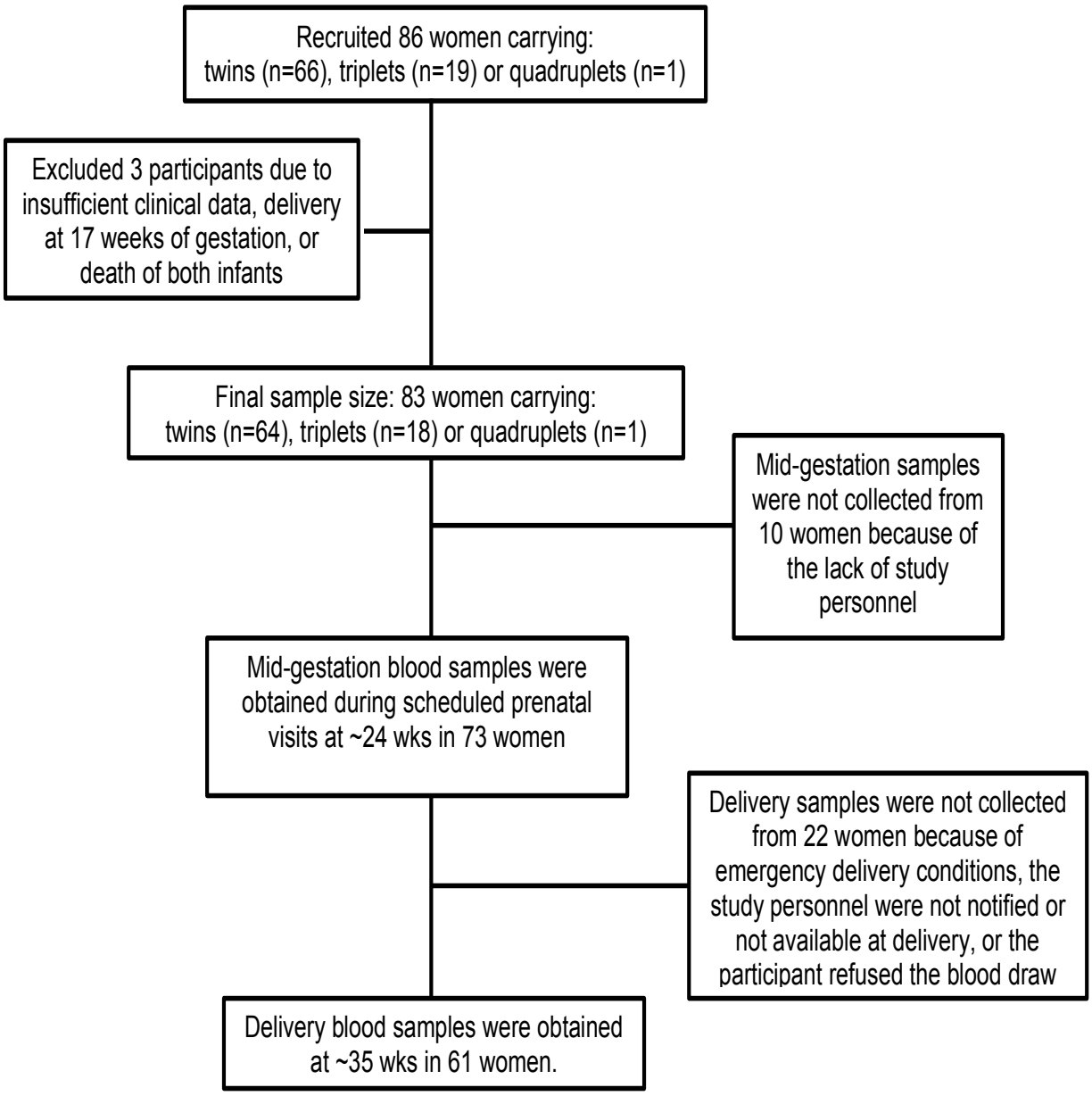
longitudinal study designed to characterize changes in maternal Fe status across gestation. All participants were recruited between 2011 and 2014 from Strong Memorial Hospital and Highland Hospital at the University of Rochester, NY. This maternity program serves as a referral center for women with multiple gestations in central and upstate New York. According to the Finger Lakes Regional Perinatal Database, 66.3% of women carrying twins and all women carrying higher-order multiples receive prenatal care at Strong Memorial Hospital and Highland Hospital. All healthy women carrying multiple fetuses (≥ 19 y) with uncomplicated pregnancies were approached and asked to participate in the study to ensure that the study population was representative of the patient population attending this clinic. Women were excluded if they had HIV infection, pre-existing diabetes mellitus, eating disorders, malabsorption diseases, hemoglobinopathies, or other medical problems known to affect Fe homeostasis at the time of enrollment. The final population included 83 women as detailed in **Figure 2.1**. Informed written consent was obtained from all participants at baseline. The study was approved by the Institutional Review Boards of the University of Rochester and Cornell University.

Demographic and health information were self-reported at study entry using a health survey questionnaire that was constructed for this study. A prenatal supplement questionnaire designed for the study was also administered at entry into the study to assess the type and frequency of prenatal supplement use. Gestational age (GA) was determined based on self-

reported menstrual history or date of in-vitro fertilization (IVF). In non-IVF patients, if GA calculated from self-reported menstrual history and that derived from the earliest ultrasound data differed by greater than 10 days, GA was determined using the earliest ultrasound estimate.

SUPPLEMENTAL FIGURE 2.1

Participant Flow Chart



Based on the World Health Organization (WHO) criteria, women were categorized as underweight, normal weight, overweight, or obese at entry into pregnancy using pre-pregnancy body mass index (ppBMI, kg/m^2) (16). A 2009 IOM committee set weight gain guidelines for twin pregnancies, but only for women who deliver at term. As the majority of our study population delivered prematurely, adequacy of gestational weight gain (GWG) was determined using the IOM guidelines adjusting for the gestational age at delivery (17). Due to the lack of weight gain guidelines for underweight women carrying multiple fetuses, underweight women (n=4) were categorized using the same IOM guidelines established for normal weight women. The GWG of women carrying triplets or quadruplets were also categorized using the same guidelines for twins due to a lack of GWG criteria for higher-order multiples. Other maternal data including gestational diabetes mellitus, pregnancy-induced hypertension, and mode of delivery were abstracted from medical records. All collected data were entered and checked by trained study personnel.

Maternal Serum Collection and Biochemical Analyses

Maternal non-fasting blood (15 mL) was obtained for study purposes during a scheduled prenatal visit and/or at delivery. In these research samples whole blood was used to assess maternal hemoglobin (Hb) and serum was separated and stored at -80^{o}C for subsequent analysis of Fe status indicators, key inflammatory markers, and serum folate and vitamin B-12. All serum samples were assayed in duplicate.

Additional longitudinal data on Hb concentrations across gestation were abstracted from medical charts as Hb was routinely assessed as standard of care when blood was drawn for alpha-fetoprotein testing at 16-20 weeks of gestation and again when oral glucose tolerance was evaluated at 26-28 weeks of gestation. Fe status indicators were only measured in the blood obtained for research purposes.

All Hb measures were analyzed using the Cell Dyn 4000 system (Abbott Laboratories, Abbott Park, Illinois) and categorized according to the trimester at which the measurement was obtained. Anemia was characterized using CDC-trimester-specific definitions as Hb < 11.0 g/dL in the 1^{st} and 3^{rd} trimester, and Hb < 10.5 g/dL in the 2^{nd} trimester (18). The severity of anemia was also categorized in all women using WHO criteria as severe (Hb < 7.0 g/dL), moderate (Hb = 7.0-9.9 g/dL), or mild anemia (Hb = 10.0-10.9 g/dL) (19). We adjusted the cut-off for anemia downwards for African Americans in our analyses based on the IOM recommendations as 10.2 g/dL for the 1^{st} and 3^{rd} trimester and 9.7 g/dL for the 2^{nd} trimester (20).

Serum ferritin (SF) and soluble transferrin receptor (sTfR) were measured using an enzyme-linked immunosorbent assay (ELISA) (Ramco Laboratories, Inc). Depleted Fe stores were defined as a SF concentration was < 12.0 µg/L. Tissue ID was defined when sTfR was > 8.5 mg/L, which has been found to have a high specificity for detecting tissue ID during pregnancy (18-20). Total body iron (TBI) was calculated using the published equation TBI (mg/kg) = $-[\log_{10}$ (sTfR x 1000/ferritin)-2.8229]/0.1207], which has been utilized

in pregnant women (1, 21), with TBI was < 0 mg/kg being defined as depleted body Fe. Women with abnormal values for any of the Fe status indicators (i.e. depleted Fe stores, tissue ID or depleted body Fe) were defined as ID. Iron deficiency anemia (IDA) was defined as presence of ID (based on SF, sTfR, or TBI) and anemia.

Serum hepcidin concentrations were analyzed using an enzyme immunoassay (EIA) kit (Bachem, CA). Values under 0.39 ng/mL were categorized as undetectable and in these samples a value of 0.195 ng/mL was assigned for analysis purposes. A pooled control serum sample was run in each assay as an internal control and the coefficient of variation (CV) for this sample was 12.2%. Serum Fe was measured using graphite furnace atomic absorption spectrophotometry (Perkin Elmer AAnalyst 800). A National Bureau of Standards reference control of bovine liver serum (Standard Reference Material® 1577c) was used as a control and values for this standard were within 5.0% of the certified expected value. Serum C-reactive protein (CRP) concentrations were measured using a quantikine ELISA kit (R&D System, MN). Values above 5 mg/L were defined as high. Serum IL-6 concentrations were assessed using a high sensitivity quantikine ELISA kit (R&D System, MN). Erythropoietin (EPO), folate and vitamin B-12 were analyzed using a Siemens Immulite® 2000 immunoassay system. Folate and vitamin B-12 insufficiency were classified when concentrations were < 6.8 nmol/L and < 148 pmol/L respectively (22).

Following current guidelines, as part of their regular prenatal care all

study participants were prescribed the same standard prenatal supplement as recommended for women carrying singletons. Women identified as being anemic were generally prescribed additional Fe supplements (325 mg of ferrous sulfate tablets to be taken twice per day for a total of 130 mg of supplemental Fe daily). Once additional Fe was prescribed, it was continued over the remainder of pregnancy as this is the standard of practice for anemic women in this clinic in accordance with national recommendations (60-120 mg Fe/day) (20). In anemic women who were prescribed additional Fe supplements, no information was obtained on the use or compliance with these recommendations.

Statistical Analysis

Normality of distributions for each continuous study measure was tested using the Shapiro-Wilk test. Non-normally distributed data were logarithmic transformed and the geometric means (GM) were calculated. Student's t test and ANOVA were used to test the normally distributed Fe indicators as a function of maternal characteristics, such as age, race, ethnicity, parity, ppBMI, and adequacy of GWG. The Wilcoxon rank sum test was used to test for statistical differences between the nonparametric variables and the Chi-square test of independence was used for analyses of the categorical variables. Bivariate associations between the Fe status indicators were plotted and assessed by Pearson correlation. Multivariate regression models were built to assess the associations between each Fe

status indicator and Hb concentration during pregnancy (~24 wks of gestation) and at delivery. Potential confounders (i.e. maternal race, gestational age and maternal parity) that have been shown to affect maternal hemoglobin concentration and Fe status were included in the models as covariates. Most data were analyzed cross-sectionally in the group as a whole. Longitudinal data analysis was also performed in the subset of women who provided multiple blood samples for research measures across gestation. In all longitudinal analyses, a mixed model with random coefficients was built to control for the lack of independence of observations due to repeated measures over time from the same mother. Logistic regression models were used to identify potential risk factors for maternal ID. A segmented regression model was used to determine the cut-off value of IL-6 at delivery where hepcidin concentrations started to rise. A sample size of 83 participants was determined to provide sufficient power (0.80) to characterize Fe status in this population and to obtain correlations among maternal Fe status indicators and inflammatory makers on the order of 0.15-0.20, with an alpha level of 0.05. Results of statistical tests were considered significant at p-values < 0.05. All statistical analyses were performed using JMP 10.0 (SAS Institute Inc, Cary, NC).

RESULTS

Maternal Characteristics

The characteristics of women enrolled in the study are presented in

Table 2.1. In those using ARTs (n=37), 48.6% (n=18) of women used intrauterine insemination (IUI), 37.8% (n=14) used IVF, and 13.5% (n=5) used clomiphene. Additionally, three women received an egg donation; 5 women received sperm donations; and 1 woman received both. Women using ARTs (n=27) had significantly higher SF (32.4 [95%CI: 23.0, 45.5] µg/L vs. 16.4 [95%CI: 12.9, 20.8] µg/L, P=0.001) and lower sTfR (4.1 [95%CI: 3.3, 5.1] mg/L vs. 5.7 [95%CI: 4.7, 6.9] mg/L, P=0.03) than their counterparts in the non-ART group (n=34) at delivery. Compared to women in the non-ART group, those in the ART group were older at delivery (33 ± 4.4 y, n=37 vs. 28 ± 4.5 y, n=46; P<0.0001), entered prenatal care earlier in gestation (6.3 ± 2.8 wks, n=35 vs. 7.6 ± 2.6 wks, n=43; P=0.05), and all were married and non-smokers.

TABLE 2.1. Baseline Characteristics of Women with Multiple Gestations[1,2]

Maternal Characteristics	Entire Cohort	Pregnancy Blood Only[3]	Delivery Blood Only[4]	Pregnancy and Delivery Blood[5]	Medical Chart Hemoglobin Data Only[6]
N	83	20	8	53	2
Maternal age at entry into prenatal care (y)	30.3 ± 5.1	30.4 ± 5.4	29.9 ± 5.9	30.6 ± 4.8	22 ± 0
Gestational age at entry into prenatal care (wks)	19.1 ± 5.6	19.4 ± 4.5	21.5 ± 7.4	18.7 ± 5.8	19.5 ± 0.7
Race					
Caucasian, %	75	85	50	75	50
African-American, %	25	15	50	25	50
Ethnicity					
Hispanic, %	6	5	13	6	50
Non-Hispanic, %	94	95	87	94	50

Parity ≥ 1, %	59	55	75	62	50
Pre-pregnancy BMI (kg/m^2)	28.2 ± 8.1	31.7 ± 8,7	24.1 ± 5.7	27.8 ± 7.8	18.8 ± 2.3
Underweight, %	5	0	0	6	50
Normal, %	42	30	75	41.5	50
Overweight, %	20	20	0	24.5	0
Obese, %	33	50	25	28	0
Gestational weight gain (kg)	19.6 ± 9.2	18.4 ± 11.9	21.6 ± 8.3	19.3 ± 8.4	14.5 ± 1.9
< Recommended, %	33	35	25	30	100
Recommended, %	43	35	50	47	0
>Recommended, %	24	30	25	23	0
Use of ART, %	45	50	25	47	0
Gestational age at delivery	34.8 ± 2.7	33.2 ± 3.4	36.6 ± 1.6	35.2 ± 2.3	34 ± 2.8
Type of Multiples					
Twins, %	77.1	60	100	79	100
Triplets, %	21.7	35	0	21	0
Quadruplets, %	1.2	5	0	0	0

[1]Data are presented as mean ± SD or the percentage. No significant differences were evident in baseline characteristics between groups (comparisons were not made for the 2 participants in the "Medical Chart Hemoglobin Data Only" cohort)

[2]Abbreviations used: BMI = body mass index; ART = assisted reproductive technologies

[3]Participants who provided serum samples only during pregnancy (24.4 ± 5.4 wks)

[4]Participants who provided serum samples only at delivery (34.8 ± 2.7 wks)

[5]Participants who provided serum samples during pregnancy and at delivery

[6]Participants who only had hemoglobin data abstracted from medical charts

Self-reported data on the frequency of supplement use was available for 88% (n=73) of the population at entry into the study (19.1 ± 5.6 wks). Of these women, 82% (n=60) stated that they consumed their supplements daily, 11% (n=8) reported that they consumed these 2-5 times per week, and 5.4% reported that they either consumed their supplements once a week (n=2) or occasionally (n=2). Due to variation in brands of prenatal supplements that were used, supplemental Fe intake ranged from 27-90 mg/day. Only one woman reported that she did not consume any prenatal supplements.

Based on self-reported ppBMI data, overweight and obesity were evident in 20% (n=17) and 33% (n=27) of the study population, respectively. The prevalence of obesity in these women prior to pregnancy is comparable to the 36.1% reported in the 2011-2012 National Health and Nutrition Examination Survey (NHANES) (23). Pre-eclampsia and pregnancy-induced hypertension occurred in 22% (n=18) of these pregnancies. Four women developed gestational diabetes during pregnancy. The majority of women carrying twins (64% n=41) delivered by Cesarean section. This prevalence is slightly lower than the 2008 national prevalence (75%) among women carrying twins (24). In this study, all but one of the 18 women carrying triplets and the participant carrying quadruplets delivered by Cesarean section. Premature delivery was prevalent in this cohort as nearly 65% (n=54) of women delivered before 37 weeks of gestation. Of those delivering preterm, 16.7% (n=9) had very preterm births (< week 32 of gestation) and 1 woman had a set of extremely preterm triplets (< week 28 of gestation). The majority of neonates

(71.5%, n=133) born to these women had low birth weight (< 2500 g) and 9.7% (n=18) were born with very low birth weight (< 1500 g). Neonatal mortality rate in this cohort was 1.6% (3 per 186 live births).

Longitudinal Changes in Iron Status across Gestation and at Delivery

Data on Fe status and inflammatory indicators were obtained in research samples collected during pregnancy and at delivery and these data are presented in **Table 2.2**. Overall 73 women provided blood samples during pregnancy, which were obtained on average at 24.4 ± 5.4 wks of gestation. Among these 73 women, 6 women had research blood drawn during the 1st trimester (11.0 ± 1.9 wks), 49 women had blood drawn during the 2nd trimester (24.2 ± 3.0 wks), and 18 women had blood drawn during the 3rd trimester (29.5 ± 2.2 wks). Delivery blood samples (35.3 ± 2.3 wks) were collected from 73.5% of women (n=61).

TABLE 2.2. Iron Status Indicators and Hemoglobin Concentrations in Blood Samples Obtained for Research Purposes in Women with Multiple Gestations[1,2]

Maternal Characteristics	Pregnancy[3](24.4 ± 5.4 wks)	Delivery (35.3 ± 2.3 wks)	P
Hemoglobin g/dL (n)	11.2 ± 1.3 (7.1-13.8) (73)	11.2 ± 1.5 (7.8-13.6) (61)	0.83
Anemia, % (n)	31.5 (23/73)	42.6 (26/61)	0.18
Anemia (Adj)[5], %(n)	27.4 (20/73)	34.3 (21/61)	0.38
sTfR, mg/L (n)	4.1 [3.7, 4.7] (73)	4.9 [4.3, 5.7] (61)	0.06

Tissue ID, sTfR>8.5 mg/L, % (n)	9.6 (27/73)	23.0 (14/61)	0.03
Ferritin, µg/L (n)	15.2 [12.9, 17.9] (73)	22.2 [17.9, 27.4] (61)	0.005
Depleted Fe stores, SF<12 µg/L, % (n)	37.0 (27/73)	26.2 (16/61)	0.18
TBI, mg/kg (n)	3.2 ± 3.4 (-5.1-10.4) (73)	3.9 ± 4.3 (-6.3-12) (61)	0.3
Depleted Body Fe, TBI<0 mg/kg, % (n)	12.3 (9/73)	18 (11/61)	0.4
IDA[6], % (n)	15.1 (11/73)	18 (11/61)	0.7
Serum Fe, (mg/L) (n)	1.4 [1.2, 1.6] (72)	1.3 [1.2, 1.8] (56)	0.5
Hepcidin, ng/mL (n)	1.3 [0.9, 1.9] (73)	2.3 [1.7, 3.2] (60)	0.03
Undetectable Hepcidin, % (n)	23.3 (17)	23.3 (17)	0.001
EPO, mIU/mL (n)	30.1 [26.0, 34.9] (72)	34.7 [27.5, 43.9] (59)	0.29
IL-6, pg/mL (n)	1.5 [1.3, 1.7] (71)	5.2 [3.8, 6.9] (57)	<0.0001
IL-6>18.4 pg/mL, % (n)	0 (0)	14 (8)	<0.0001
CRP, mg/L (n)	4.5 [3.6, 5.6] (71)	4.4 [3.2, 6.0] (59)	0.85
CRP>5 mg/L, % (n)	45.1 (32)	42.4 (25)	0.76
Folate, nmol/L (n)	NA[7]	22 [18.8, 25.5] (55)	
Folate<6.8 nmol/L, % (n)	NA	0 (0)	
Vitamin B-12, pmol/L, (n)	NA	405.9 [359.2, 458.7] (47)	
Vitamin B-12<148 pmol/L, % (n)	NA	0 (0)	

[1]Data are presented as the mean ± SD (Range), the geometric mean [95% CI], or the percentage, with the sample size in parentheses; P value reported

based on statistical differences between pregnancy and delivery (2-tailed t test or chi square test)

[2]Abbreviations used: sTfR = soluble transferrin receptor; Tissue ID = tissue iron deficiency; TBI = total body iron; EPO = erythropoietin; CRP = C-reactive protein

[3]Pregnancy blood samples were obtained for research purposes across all three trimesters

[4]Anemia defined as hemoglobin (Hb) < 11.0 g/dL in the 1st and 3rd trimesters; Hb < 10.5 g/dL in the 2nd trimester

[5]Anemia (Adj.) was adjusted for race. Anemia for African American women defined as Hb < 10.2 g/dL in the 1st and 3rd trimesters; Hb < 9.7 g/dL in the 2nd trimester

[6]Iron deficiency anemia (IDA) was defined as presence of anemia (Adj.) and ID (tissue ID, depleted Fe stores, or depleted body Fe)

[7]NA = No data were available because these indicators were not analyzed in the pregnancy samples

The prevalence of tissue ID (sTfR > 8.5 mg/L) increased significantly from 9.6% (n=73) during pregnancy (24.4 ± 5.4 wks) to 23% (n=61) at delivery (P=0.03). Of the 73 women who provided blood samples during pregnancy, 37% (n=27) had depleted Fe stores (SF<12 µg/L) while receiving standard prenatal supplementation. The prevalence of depleted Fe stores in our population was significantly higher than the prevalence reported for similar trimesters of

pregnancy in the NHANES 1999-2006 data during the 1st [33.3% (n=2/6) vs. 7.3%, P= 0.007] and the 2nd trimester [40.8% (n=20/49) vs. 23.7%, P=0.01], respectively (1).

The prevalence of IDA in this population was 15.1% (n=73) at 24.4 ± 5.4 weeks of gestation and did not change significantly from pregnancy to delivery (18% at 35.3 ± 2.3 weeks of gestation, n=61). This lack of change between the two sampling points might be due in part to the fact that premature delivery was common in these women, the delivery Hb measures were taken on average 11 weeks after the pregnancy sample and Hb concentrations are known to increase slightly in late gestation (25). Published NHANES data on Fe status across gestation presented data on ID but did not provide data on IDA so we cannot evaluate our IDA data in relation to the national NHANES data (1)

All women studied had detectable IL-6 and CRP during pregnancy and at delivery. While maternal CRP concentrations did not change significantly between pregnancy and delivery, maternal IL-6 concentrations increased significantly across gestation and were significantly associated with both serum ferritin and hepcidin at delivery only **(See Tables 2.4 & 2.5)**. Nearly 10% of the variance in the change in hepcidin from pregnancy to delivery was explained by IL-6. Using a segmented regression model, we found that at delivery, hepcidin concentrations increased markedly when IL-6 concentrations reached 18.4 pg/mL.

TABLE 2.3. Associations Between Hemoglobin, Iron Status Indicators, and Inflammatory Markers during Pregnancy[1]

	Log SF	Log sTfR	TBI	Log Serum Fe	Log EPO	Log Hepcidin	Log IL-6	Log CRP
Hb	**r=0.26** **P=0.03** **N=72**	r=-0.20 P=0.09 N=72	**r=0.31** **P=0.009** **N=72**	**r=0.27** **P=0.02** **N=71**	**r=-0.54** **P<0.0001** **N=71**	**r=0.27** **P=0.02** **N=72**	r=0.10 P=0.42 N=70	r=0.04 P=0.76 N=71
Log SF		r=-0.20 P=0.09 N=73	**r=0.85** **P<0.0001** **N=73**	**r=0.25** **P=0.04** **N=72**	r=-0.19 P=0.10 N=72	**r=0.43** **P=0.0002** **N=73**	r=0.09 P=047 N=71	r=0.03 P=0.82 N=71
Log sTfR			**r=-0.69** **P<0.0001** **N=73**	r=-0.08 P=0.49 N=72	**r=0.42** **P=0.0002** **N=72**	**r=-0.25** **P=0.03** **N=73**	r=0.13 P=0.28 N=71	r=0.06 P0.60 N=71
TBI				r=0.23 P=0.06 N=72	**r=-0.37** **P=0.01** **N=72**	**r=0.45** **P<0.0001** **N=73**	r=0.006 P=0.96 N=71	r=-0.01 P=0.91 N=71
Log Serum Fe					r=-0.06 P=0.6 N=71	**r=0.25** **P=0.03** **N=72**	r=-0.03 P=0.83 N=70	r=-0.05 P=0.65 N=70
Log EPO						**r=-0.38** **P=0.001** **N=72**	r=0.16 P=0.18 N=70	r=-0.09 P=0.45 N=70
Log Hepcidin							r=0.13 P=0.27 N=71	**r=0.30** **P=0.01** **n=71**
Log IL-6								**r=0.60** **P<0.0001** **N=70**

[1]Blood samples were taken on average at 24.4 ± 5.4 weeks of gestation

[2]Associations that are significant (Pearson correlation) are presented in bold font, P<0.05

TABLE 2.4. Associations Between Hemoglobin, Iron Status Indicators, and Inflammatory Markers at Delivery[1]

	Log SF	Log sTfR	TBI	Log Serum Fe	Log EPO	Log Hepcidin	Log IL-6	Log CRP
Hb	**r=0.42 P=0.0008 N=61**	**r=-0.42 P=0.0008 N=61**	**r=0.48 P<0.0001 N=61**	**r=0.41 P=0.002 N=58**	**r=-0.50 P<0.0001 N=59**	**r=0.32 P=0.01 N=60**	r=0.10 P=0.48 N=57	r=0.04 P=0.74 N=59
Log SF		**r=-0.47 P=0.0001 N=61**	**r=0.91 P<0.0001 N=61**	**r=0.32 P=0.01 N=58**	**r=-0.47 P=0.0002 N=59**	**r=0.60 P<0.0001 N=60**	**r=0.30 P=0.02 N=57**	r=0.25 P=0.06 N=58
Log sTfR			**r=-0.79 P<0.0001 N=61**	**r=-0.33 P=0.01 N=58**	**r=0.62 P<0.0001 N=59**	**r=-0.46 P=0.0002 N=60**	r=0.13 P=0.33 N=57	r=-0.25 P=0.06 N=58
TBI				**r=-0.38 P=0.004 N=58**	**r=-0.61 P<0.0001 N=59**	**r=0.63 P<0.0001 N=60**	**r=0.27 P=0.04 N=57**	**r=0.29 P=0.03 N=58**
Log Serum Fe					**r=-0.32 P=0.01 N=57**	r=-0.02 P=0.86 N=57	r=-0.16 P=0.25 N=55	r=-0.005 P=0.97 N=57
Log EPO						**r=-0.29 P=0.03 N=58**	r=0.03 P=0.85 N=56	r=0.01 P=0.93 N=57
Log Hepcidin							**r=0.38 P=0.004 N=56**	**r=0.41 P=0.001 n=57**
Log IL-6								**r=0.64 P<0.0001 N=70**

[1]Blood samples were taken on average at 35.3 ± 2.3 weeks of gestation

[2]Associations that are significant (Pearson correlation) are presented in bold font, P<0.05

Longitudinal Changes in Hemoglobin across Gestation and at Delivery

Mean Hb concentrations and the prevalence of anemia during each trimester are presented in **Table 2.5**. Data in **Tables 2.2 and 2.5** were obtained from blood samples taken for research purposes. For Table 3, when Hb data were available from both the medical charts and from the research blood sample, only the research values were reported. The prevalence of anemia increased significantly from the 1st trimester to the 3rd trimester (8.1% vs. 45.5% P<0.0001). Early anemia was predictive of anemia during later gestation as evidenced by the finding that Hb measures obtained during either the 1st (R^2=0.08, P=0.01, n=62) or 2nd (R^2=0.38, P<0.0001, n=72) trimester were significantly positively associated with Hb concentrations at delivery. Women who were anemic during the 2nd trimester had a nearly 2-fold increased risk of anemia (RR=1.9 [95% CI: 1.1, 3.2]) at delivery after adjusting for race and gestational week at delivery. During the 2nd and 3rd trimester, 31.9% (n=72) and 45.5% (n=77) of women were anemic, respectively. This prevalence of anemia was significantly higher than national NHANES data (1999-2006) in which 2.2% (P<0.0001) and 10.8% (P<0.0001) of women carrying singletons had anemia in the 2nd and 3rd trimester, respectively (1).

TABLE 2.5. Hemoglobin Concentrations and Prevalence of Anemia in Women with Multiple Gestations Using Both Blood Samples Obtained for Research Purposes and Data Abstracted from Medical Charts [1,2]

	Trimester 1 10.3 ± 1.7 wks	Trimester 2 23.5 ± 4.0 wks	Trimester 3 30 ± 2.5 wks	Delivery 34.8 ± 2.7 wks	P
Entire Cohort					
N	62	72	77	83	
Hb (g/dL)	12.3 ± 1.0[a] (10 -14.2)	10.9 ± 1.4[b] (7.5 -13.7)	11.1 ± 1.6[b] (7.1-14)	11.2 ± 1.6[b] (7.7-13.9)	<0.0001
Anemia[3], % (n)	8.1[a] (5/62)	31.9[b] (23/72)	45.5[b] (35/77)	44.6[b] (37/83)	<0.0001
Severe[4], % (n)	0 (0/62)	0 (0/72)	0 (0/77)	0 (0/83)	
Moderate[4], % (n)	0 (0/62)	18 (13/72)	23.4 (18/77)	21.7 (18/83)	0.54
Mild[4], % (n)	8.1[a] (5/62)	13.9[b] (10/72)	22.1[b] (17/77)	22.9[b] (19/83)	0.01
IDA, % (n)[5]	16.7 (1/6)	14.3 (7/49)	22.1[b] (17/77)	22.9[b] (19/83)	0.64
Caucasian Women					
N	48	54	57	62	
Hb (g/dL)	12.3 ± 1.0[a] (10 -14.2)	11.2 ± 1.3[b] (7.7-13.7)	11.4 ± 1.4[b] (8.6 -14)	11.5 ± 1.4[b] (8.8 -13.9)	<0.0001
Anemia[3], % (n)	10.4[a] (5/48)	25.9[b] (14/54)	35.1[b] (20/57)	32.3[b] (20/62)	0.02
IDA, % (n)[5]	20 (1/5)	17.9 (7/39)	23.1 (3/13)	15.9 (7/44)	0.46
African-American Women					
N	14	18	20	21	
Hb in African-Americans (g/dL)	12.2 ± 0.7[a] (11.4 -13.7)	10.3 ± 1.3[b] (7.5 – 12)	10 ± 1.5[b] (7.1 – 13.4)	10.2 ± 1.6[b] (7.7 – 13.5)	<0.0001
Anemia, % (n)	0 (0/14)	50 (9/18)	75 (15/20)	81 (17/21)	0.09

Anemia (Adj.)[3], % (n)	0 (0/14)	16.7[a] (3/18)	50[b] (10/20)	52.4[b] (11/21)	0.04
IDA, % (n)[5]	0 (0/1)	0 (0/10)	0 (0/5)	23.5 (4/17)	0.23

[1]For any given trimester, when Hb data were available from both the medical charts and from the research blood, only the research value was reported. Data are presented as the mean ± SD with the range or the percentage with the sample size presented in parentheses

[2]Different superscripts in the table indicate significant differences between different trimesters, (P<0.05, ANOVA or chi-square test)

[3]Anemia defined as hemoglobin (Hb) < 11.0 g/dL in the 1st and 3rd trimesters; Hb < 10.5 g/dL in the 2nd trimester; Anemia (Adj.) adjusted for race (-0.8 g/dL) for African-American women

[4]Severe anemia: Hb < 7.0 g/dL; moderate anemia: Hb = 7.0-9.9 g/dL; mild anemia: Hb = 10.0-10.9 g/dL

[5]Iron deficiency anemia (IDA) is defined as the presence of anemia and ID (based on SF, sTfR, or TBI). Sample size presented is the number of women with IDA out of the total number of women who had both Hb and Fe status indicator data available within each time period presented. Gestational week of IDA: 1st trimester (11.0 ± 1.9 wks, n=6), 2nd trimester (24.2 ± 3.0 wks, n=49), 3rd trimester (29.5 ± 2.2 wks, n=18), and delivery (35.3 ± 2.3 wks, n=61)

In the 31.9% of women (23 of 72 studied) who were anemic when studied during the 2nd trimester (23.5 ± 4.0 wks), 73.9% (n=17) remained anemic at delivery (34.2 ± 3.4 wks). In the 45.5% of women (35 of 77 studied) who were anemic during the 3rd trimester (30 ± 2.5 wks), 82.9% (n=29) remained anemic at delivery (34.7 ± 3.3 wks). To further explore longitudinal changes in maternal hemoglobin concentrations across pregnancy at the individual level, a random intercept and slope model was applied in women with repeat measures (n=83). Maternal hemoglobin concentrations decreased significantly as pregnancy progressed (β=-0.02, SE=0.008, P=0.01). A quadratic relationship was observed between maternal hemoglobin and the gestational stage of pregnancy indicating that the rate of decline in hemoglobin was fastest during the earlier stages of pregnancy (week 5-28) and reversed during late pregnancy (after week 30) (**Figure 2.1**). Using the IOM race-adjusted definitions of anemia (20), the prevalence of anemia among African Americans was 1.4-fold and 1.6-fold higher than that observed among Caucasian women during the 3nd trimester and at delivery, respectively.

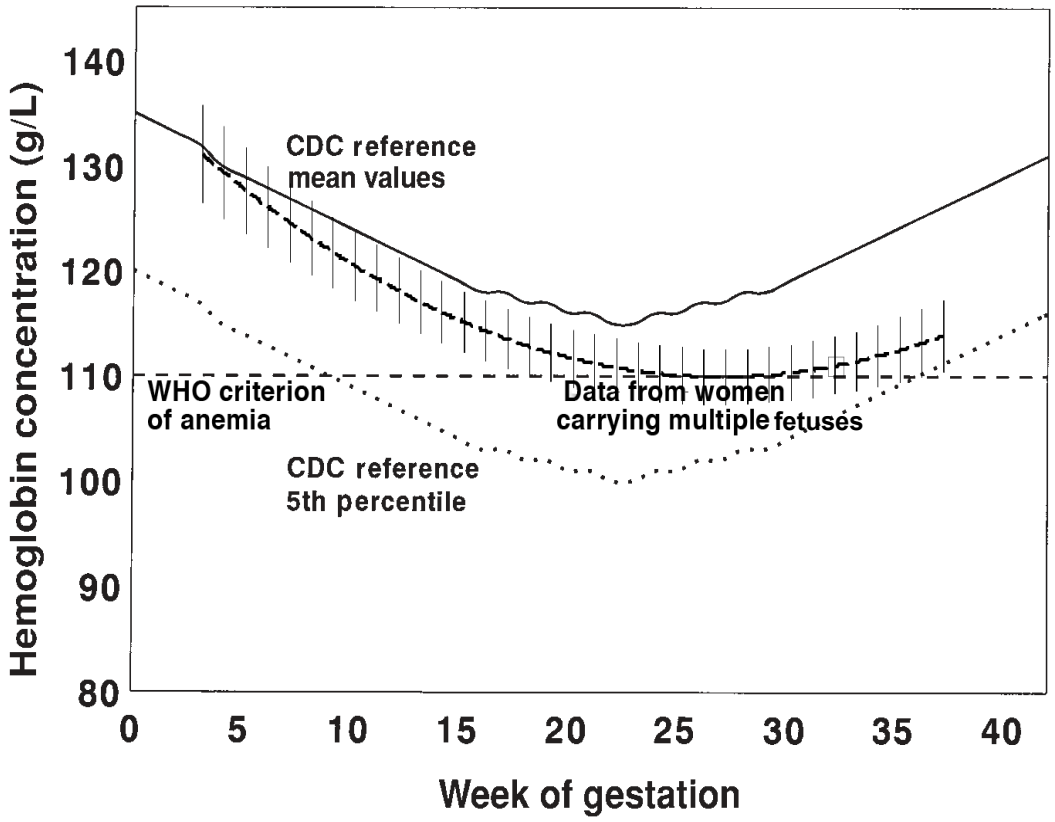


FIGURE 2.2 LEGEND

Figure 1. Longitudinal changes in hemoglobin concentrations across pregnancy in 83 women carrying multiple fetuses (dashed black line with SD) were compared to CDC reference mean values (solid black line). The dotted line represents the 5th percentile of the CDC reference data. A quadratic relationship between hemoglobin and gestational week was found in these women carrying multiple fetuses using a non-linear regression model. Maternal hemoglobin concentrations decreased significantly as pregnancy progressed (β=-0.02, SE=0.008, p=0.01) with the rate of decline being steepest during the earlier stage of pregnancy (week 5-25 of gestation) and then reversing slightly during late pregnancy (>30 wks of gestation). Figure 1

was based on the original data obtained from 1989 CDC Morbidity and Mortality Weekly Report (170) and adapted from Beaton et al (169).

Determinants of Hemoglobin and Iron Status At Delivery

Among those with depleted Fe stores (SF<12 µg/L) during pregnancy (24.4 ± 5.4 wks, n=27), 74% (n=20) had both Hb and Fe status indicator data (SF, sTfR, and TBI) available at delivery. Of these 20 women, 25% (n=5) developed IDA and 65% (n=13) were anemic at delivery. Women with depleted Fe stores during pregnancy had a 2-fold greater risk of anemia at delivery (RR=2.0 [95% CI: 1.1, 3.8]). EPO concentrations measured during pregnancy (at ~ 24 wks) were significantly negatively associated with maternal Hb at delivery (~ 35 wks) after adjusting for maternal race, gestational age, and maternal parity (R^2=0.18, P=0.005, n=72). Women who had an EPO concentration during pregnancy above the 75th percentile (49.7 mIU/mL, n=16) exhibited a 3-fold higher risk of anemia at delivery (RR=3.0 [95% CI: 1.7, 5.3]). EPO was also significantly negatively associated with SF, hepcidin, and serum Fe, and positively associated with sTfR at delivery **(Supplemental Table 2.2)**. EPO alone explained 28%, 24%, and 14% of the change in sTfR, Hb, and SF from pregnancy to delivery, respectively. Unlike ferritin and hepcidin, EPO concentrations were not confounded by inflammation since EPO was not associated with either IL-6 or CRP during pregnancy or at delivery.

Parity was significantly associated with maternal Fe status at delivery. At delivery, women with a parity ≥ 1 (n=34) had significantly lower SF (18.1 [95%

CI: 14.0, 23.4] µg/L vs. 31.7 [95% CI: 22.6, 44.6] µg/L, P=0.01) and hepcidin (1.6 [95% CI: 1.2, 2.4] ng/mL vs. 4.2 [95% CI: 2.3, 7.6] ng/mL, P=0.007), and significantly higher sTfR (5.9 [95% CI: 5.0, 6.9] mg/L vs. 3.6 [95% CI: 2.8, 4.7] mg/L, P=0.002) when compared to the primagravida (n=49). In addition, marital status was significantly associated with Hb and Fe status at delivery. Single women had significantly lower Hb concentrations (10.6 ± 1.6 g/dL, n=30 vs. 11.6 ± 1.5 g/dL, n=50, P=0.006), lower SF (16.8 [95% CI: 12.2, 23.2], n=22 µg/L vs. 27.3 [95% CI: 20.7, 35.9] µg/L, n=36, P=0.03), lower hepcidin (1.3 [95% CI: 0.9, 2.3] ng/mL, n=22 vs. 3.6 [95% CI: 2.4, 5.4] ng/mL, n=35, P=0.004), and higher EPO (48.4 [95% CI: 35.2, 66.5] MIU/mL, n=21 vs. 27.4 [95% CI: 20.1, 37.4] MIU/mL, n=35, P=0.02) than their married counterparts at delivery. Hemoglobin, sTfR and SF concentrations at delivery were not significantly associated with maternal age at delivery, ethnicity, or self-reported prenatal supplement use.

2.5 DISCUSSION

To the best of our knowledge, this is the largest study to explore maternal ID and anemia in women carrying multiple fetuses. Both ID and anemia were prevalent in this high-risk maternity population. Anemia was found in 45% of women during the 3rd trimester, 4-fold higher than the expected national prevalence reported for women carrying singletons (1). Although early ID was a significant determinant of anemia at delivery in women with multiple gestations, there are no early screening guidelines in this

group and they are not among the high-risk groups identified by the last published ACOG Practice Bulletin on anemia in pregnancy (15). Erythropoietin was found to be the strongest determinant of maternal Hb at delivery. Given the high correlations found between EPO during pregnancy and Hb at delivery, and the findings that EPO was not impacted by maternal inflammation, screening for elevated EPO during pregnancy may help to identify women who are at higher risk of developing anemia at delivery.

Maternal ID was prevalent in women with multiple gestations with tissue ID increasing significantly from pregnancy to delivery. A significantly higher prevalence of depleted Fe stores was found during the 1st and 2nd trimesters when compared to 1999-2006 NHANES data (1). Of women with depleted Fe stores during pregnancy, more than half were found to be anemic ~10 weeks later when they delivered their infants. It should be noted that similar to national data in (9), 65% (n=54) of our population delivered prematurely and the high prevalence of anemia and ID noted at delivery were found roughly 5 weeks earlier than a typical term gestation (40 wks). Anemia is known to increase the risk of PTB and LBW (2), adverse birth outcomes that are highly prevalent in women carrying multiple fetuses. The degree to which maternal anemia exacerbates risk of PTB and LBW in this group is unknown and merits further study.

Nearly half of this study population was anemic at delivery. Prevalence of anemia during the 2nd (31.9%, n=23) and 3rd (45.5%, n=35) trimesters was significantly higher than national NHANES data (1999-2006) in women

carrying singletons (1). Few normative Hb data exist for women carrying multiple fetuses. In a retrospective, case-control study matched for parity, significantly lower Hb concentrations were evident during the 1st and 2nd trimester in twin vs. singleton pregnancies (11). In our population, changes in Hb across gestation followed a quadratic relationship with the greatest decline being evident during early gestation and a nadir occurring around 28 weeks of gestation at which time maternal plasma volume has been reported to be increased by 50%-100% in women with multiple gestations (26). Compared to the CDC reference curve based on iron-supplemented singleton pregnancies (25, 27), the Hb curve in our study population was shifted downwards at all gestational ages, with a markedly steeper decrease in early gestation.

The observed prevalence of IDA at delivery was 18%, but this accounted for only 30% of overall anemia observed. Folate and vitamin B-12 deficiencies were not evident in this population. Growing data in pregnant and non-pregnant populations are linking deficiencies of vitamin A (28), selenium (29), and vitamin D (30), to risk of anemia. Further studies are needed to explore other causes of anemia in those without ID. Normative data on IDA in pregnant women were not reported in the NHANES (1) or CDC (31) studies thus we were unable to compare our IDA prevalence to national data.

EPO was the only Fe biomarker during pregnancy that was significantly associated with Hb at delivery. EPO increases in response to anemia, ID, and hypoxia (32). In our study, women with EPO concentrations above the 75th percentile during pregnancy were 3 times more likely to be anemic at delivery.

EPO alone explained 14%-28% of the change in sTfR, Hb and SF from pregnancy to delivery. Clinical labs routinely measure serum EPO, but this indicator is not typically utilized to help identify women who are at subsequent risk of ID or anemia. Our study found EPO to be a strong determinant of Fe status and anemia at delivery and this indicator was not impacted by inflammation.

While SF is commonly used to diagnose ID, there are limitations with the use of SF under inflammatory conditions (33, 34). In our study population, IL-6 was positively associated with both SF and hepcidin at delivery, consistent with earlier findings in teen pregnancies (35). Serum IL-6 up-regulates hepcidin synthesis and this cytokine is frequently utilized when examining associations between inflammation and hepcidin. In our study, hepcidin concentrations were significantly higher in those with IL-6 > 18.4 pg/mL at delivery. Given that 92% of the study population exhibited increases in IL-6 from pregnancy to delivery, these findings highlight the need to include a marker of inflammation when interpreting Fe status indicators that also function as acute phase proteins (ferritin and hepcidin) in order to avoid underestimating the prevalence of ID.

One strength of this study is that 64% of women had longitudinal measures of Fe status across gestation in this understudied high-risk population. Our study is distinct from the previous studies on Fe status in multiples (12, 36) because of our use of multiple Fe biomarkers, assessment of inflammation, and longitudinal follow-up to delivery. Some study limitations

should be noted. We assume that women in the Central NY area that received prenatal care at Strong Memorial and Highland Hospitals (66% of multiple gestations) were similar to the remaining 34% seen at other hospitals in Central NY. We did not obtain normative data from an matched population carrying singletons for comparison purposes nor did we obtain dietary data to assess dietary determinants of maternal anemia and ID. Supplement compliance data was based on self-report and collected only at \baseline. Blood samples were obtained during scheduled prenatal visits for convenience purposes, thus the timing of these assessments was not standardized, nor were longitudinal measures available in all study participants. Finally, plasma volume determinations were not obtained and variability in blood volume expansion would impact interpretation of biomarkers during pregnancy. A U-shaped distribution between Hb at term and increased risk of adverse birth outcomes have been found, which may in part be due to altered plasma volume expansion (37-40).

In conclusion, women with multiple gestations exhibited a high prevalence of anemia and ID. Maternal ID and anemia during critical windows of pregnancy have been shown to impact fetal growth and development, and increasing data support an association between maternal ID and insufficient neonatal Fe stores at birth (41, 42). Given the highly significant correlations between Fe status early in gestation with depleted body Fe and/or anemia at delivery, early screening for maternal Fe status is warranted in this high-risk population so that early intervention and Fe supplementation regimens can be

initiated when warranted. Additional studies are needed to identify the amount of supplemental Fe that is required by women carrying multiple fetuses, to address the possible impact of maternal Fe status on neonatal Fe stores in this high-risk obstetric population, and to investigate the etiology of non-iron deficiency anemia.

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CHAPTER 3

PREDICTORS OF ANEMIA AND IRON STATUS AT BIRTH IN NEONATES BORN TO WOMEN CARRYING MULTIPLE FETUSES

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3.1 ABSTRACT

Background: Iron (Fe) status of neonates born to women carrying multiple fetuses might be compromised as a consequence of the high prevalence of maternal iron deficiency and anemia coupled with an increased risk of preterm birth in this group.

Objectives: We aimed to characterize umbilical cord iron status indicators and identify determinants of anemia in neonates born to women carrying multiple fetuses.

Design: Umbilical cord blood obtained from 183 neonates born to women carrying multiple fetuses was used to assess hemoglobin (Hb), ferritin (SF), soluble transferrin receptor (sTfR), hepcidin, serum Fe, erythropoietin, folate, vitamin B-12, C-reactive protein and interleukin-6. Associations with maternal Fe status were explored.

Result: No significant changes in cord Hb or SF were found as a function of gestational age at birth between 25-38 weeks. The prevalence of anemia was not significantly different between preterm neonates (17%) and early-term neonates (12%). Neonates born to women who were obese prior to pregnancy or smoked cigarettes during pregnancy had a 4-fold and a nearly 5-fold greater odds of having anemia at birth, respectively. Cord sTfR was the strongest indicator of cord Hb (β=2.6, P<0.0001, n=126), capturing 41% of the variance in cord Hb. Cord sTfR was significantly associated with maternal sTfR at mid-gestation (β=0.25, P=0.01, n=128) and delivery (β=0.25, P=0.002, n=120). Cord Fe indicators were significantly associated with cord hepcidin,

but not maternal hepcidin, suggesting that neonates regulate their Fe status independently from their mothers.

Conclusion: Screening for Fe status in neonates born to women carrying multiple fetuses is warranted, especially for those born to smokers or women who are obese prior to pregnancy. Further studies are needed to optimize neonatal Fe stores at birth in support of healthy outcomes.

3.2 INTRODUCTION

Multiple births currently account for nearly 3.5% of all births in the U.S. (1). The health of neonates born to women carrying multiple fetuses is of concern as this group alone is responsible for 15% of all preterm births (PTB) and 20% of all low birth weight (LBW) infants born in the U.S. (2). To date, little attention has been placed on the unique nutritional concerns of this population. Iron (Fe) in particular merits attention, given its role in early brain growth and function (3-7). Maternal anemia also significantly increases risk of LBW and PTB (8-10). Evidence suggests that neonatal Fe status may be compromised as a consequence of maternal Fe deficiency (ID) (11-13).

Fetal Fe accretion increases nearly 10-fold from 0.8 mg/day in the 1st trimester to roughly 7.5 mg/day during the 3rd trimester (14). Little is known about the ability of the pregnant gravida to modify Fe partitioning in order to supply multiple fetuses with sufficient Fe at birth. Since PTB is prevalent among women with multiple gestations and most Fe is accrued over late pregnancy, early delivery may predispose these neonates to suboptimal Fe

stores at birth. A growing literature emphasizes the unique Fe partitioning that occurs *in utero* to allow Fe to be prioritized for erythropoiesis at the cost of fetal brain and cardiac Fe requirements (13, 15, 16).

Few studies have examined the prevalence of anemia in neonates born to women carrying multiple fetuses. The latest American Academy of Pediatrics (AAP) publication on ID and Fe deficiency anemia (IDA) among infants 0-3 y of age highlighted an increased risk of anemia in preterm and small for gestational age (SGA) infants but did not mention multiples as a group at higher risk. More data in this higher risk population are needed (17).

We recently reported an increased risk of maternal anemia and ID in a large cohort of women with multiple gestations (18). Given the known associations between maternal and neonatal Fe status, the high prevalence of PTB among multiples, and the increased risk of poor neonatal Fe stores due to PTB, the goal of this study was to characterize neonatal Fe status at birth and identify significant determinants of ID and anemia in newborns born to women carrying multiples fetuses. Because anemia is the last stage of Fe depletion, additional normative data on Fe status in vulnerable groups are needed in relation to gestational age (GA) at birth and maternal Fe status across gestation.

3.3 SUBJECTS AND METHODS

Participants

Women (≥ 19 y) carrying twins, triplets, or quadruplets were recruited

between 2011 and 2014 from Strong Memorial Hospital and Highland Hospital at the University of Rochester, in Rochester, NY. Exclusion criteria included HIV infection, pre-existing diabetes, malabsorption diseases, hemoglobinopathies, or other medical problems known to affect Fe homeostasis. This study was approved by the Institutional Review Boards of the University of Rochester and Cornell University. Data on Fe status (18) and determinants of umbilical cord coiling (19) in these women have been published.

All women were prescribed standard prenatal supplements containing between 27-90 mg of Fe. GA was determined as previously described (18). Maternal pre-pregnancy body mass index (ppBMI) was characterized as underweight (<18.5 kg/m^2), normal weight (18.5-24.9 kg/m^2), overweight (25.0-29.9 kg/m^2), or obese (30.0-34.9 kg/m^2) (20). Birth weight, GA at birth, delivery mode, and Apgar scores were abstracted from medical records. Delayed cord clamping was not a standard practice for multiple gestations at the time this study was implemented. Neonates were categorized as preterm (<37 weeks) or early term (37-39 weeks) (21). Growth rate at birth was assessed using the Fenton growth charts and birth weight <10^{th} percentile was defined as small-for-gestational age (SGA) (22, 23). Discordant growth between siblings was evaluated by subtracting the smaller from the larger neonatal weight and then dividing by the larger weight (24). Discordant growth was defined as a greater than 15-25% difference in birth weight between twin, triplet, or quadruplet siblings.

A total of 186 neonates (64 sets of twins, 18 sets of triplets and 1 set of quadruplets) born to 83 women (aged 30.3 ± 5.1 y) were recruited. These 83 women had no significant difference in baseline maternal characteristics between either the entire cohort or the two sub-groups of participants that provided blood samples during pregnancy and/or at delivery (18). Three cases of neonatal mortality occurred resulting in a final study population of 183 neonates (53% were female). Umbilical cord blood samples were not collected from 39 neonates at birth due to emergency delivery conditions **(See Figure 3.1)**.

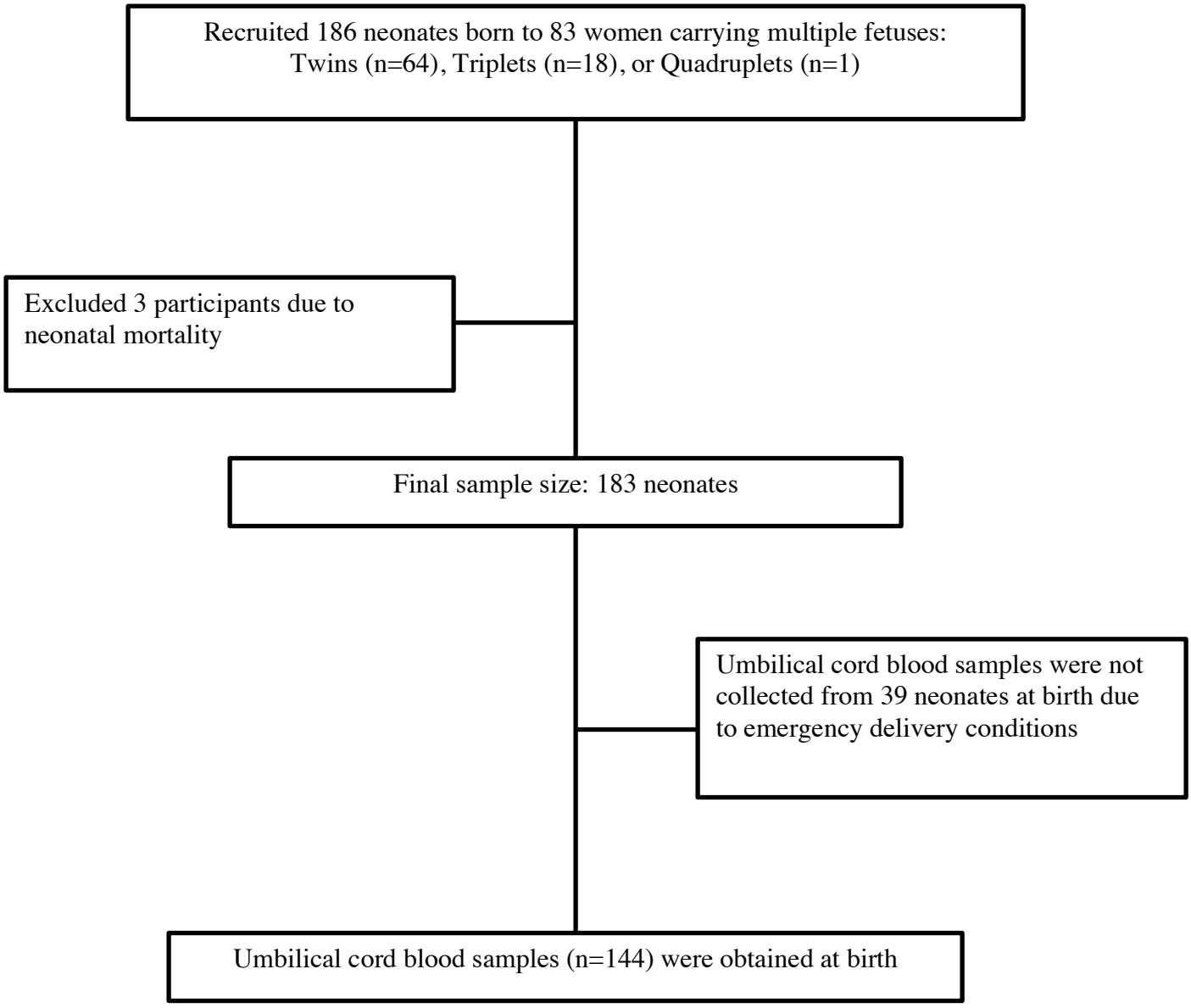

FIGURE 3.1. Participant Flow Chart

Cord Blood Collection and Biochemical Analyses

Cord hemoglobin (Hb) was measured at the Strong Memorial Hospital clinical laboratory using Cell-Dyn 4000 system (Abbott, Santa Clara, CA). Neonatal anemia was defined as a cord Hb < 13.0 g/dL using published

reference data (25). Cord serum ferritin (SF) and soluble transferrin receptor (sTfR) were analyzed using ELISAs (Ramco, Stafford, TX) with the intra- and inter-assay coefficients of variation 4.2% and 5.3% for SF, respectively, and 3.2% and 4.0% for sTfR respectively. Cord hepcidin was assessed using a hepcidin-25 enzyme immunoassay (Bachem, Torrance, CA). Hepcidin concentrations ≤ 0.39 ng/mL were categorized as undetectable and assigned a value of 0.195 ng/mL. Serum Fe was measured using graphite furnace atomic absorption spectrophotometry (Perkin Elmer AAnalyst 800). A National Bureau of Standards reference control of bovine liver serum (Standard Reference Material 1577c) was used as a control.

Cord C-reactive protein (CRP) was measured using a Quantikine ELISA kit with the intra- and inter-assay coefficients of variation 5.5% and 6.5%, respectively. Interleukin 6 (IL-6) was measured using a high sensitivity Quantikine ELISA kit (R&D System, MN) with the intra- and inter-assay coefficients of variation 7.4% and 7.7%, respectively. CRP values < 0.078 mg/L and IL-6 values < 0.156 ng/mL were below the assay limit of detection and were assigned values of 0.039 mg/L and 0.078 ng/mL, respectively. Erythropoietin (EPO), folate and vitamin B-12 were analyzed using a Siemens Immulite® 2000 immunoassay system. Insufficiency was defined when folate concentrations were < 6.8 nmol/L or vitamin B-12 concentrations were < 146 pmol/L (26). Maternal Fe and inflammation indicators were analyzed as detailed (18). All Fe status indicators and inflammatory markers were assayed in duplicate. For folate and vitamin B-12, only 15% of the samples were

assayed in duplicate due to budgetary constraints.

Statistical Analysis

Normality of distributions for each continuous study measure was tested using the Shapiro-Wilk test and means were calculated using a random effects model to control for non-independence of multiple siblings. Non-normally distributed data were logarithmic transformed and the geometric means were calculated. Gestational age, race, ethnicity, and discordant growth, were presented along with the maternal characteristics. Maternal characteristics between twins and triplets/quadruplets groups were compared using a two-tailed t-test. The Chi-square test of independence was used for analyses involving categorical variables. Because neonates born to the same mother are not independent, neonatal characteristics between twins and triplets/quadruplets groups were compared using a mixed model with random effects. The same mixed model was used between siblings to evaluate possible associations between cord and maternal Fe status indicators, and the Bonferroni test ($p<0.002$) was used to correct for multiple comparisons. A generalized linear mixed model was applied to control for the dependence of Fe status between siblings and to identify significant determinants of neonatal anemia. Potential confounders, including gestational weight gain (GWG), gestational age, and birth weight, were included in all models as covariates. The neonatal sample size was sufficient to detect correlations on the order of 0.15-0.20, with an alpha level of 0.05. All statistical analyses were performed

using JMP 12.0 (SAS Institute Inc, Cary, NC).

3.4 RESULTS

Maternal and Neonatal Characteristics

Maternal (n=83) and neonatal (n=183) characteristics are presented in **Table 3.1**. Compared to women carrying twins, as expected, women carrying higher order multiples (triplets and quadruplets) had a significantly higher incidence of preterm/very preterm delivery, cesarean section, and neonatal discordant growth. Significantly fewer African-American women delivered triplets. Based on self-reported pre-pregnancy weight, 20% of women were overweight (n=17/83) and 33% (n=27/83) were obese. Compared to twins, the triplets/quadruplet group had significantly lower mean birth weight.

In these 183 neonates, nearly 70% of neonates were born prematurely (<37 weeks) and the remainder were born early term (37-38 weeks). A quarter of the PTBs observed (n=31/124) were due to premature rupture of membranes and the remaining 36% of PTB's (n=44/124) were due to other pregnancy related complications including preeclampsia, HELLP, fetal death, intrauterine growth restriction, and peripartum cardiomyopathy, complications that can occur in multiple births. The majority of neonates studied (74%) were delivered by cesarean section. Twin to twin transfusion syndrome was found in a set of monochorionic and a set of diamniotic twins and in both instances this was successfully treated by laser surgery early in pregnancy. Neonatal birth weight averaged 2,206.5 ± 571.5 g, 71% of neonates (n=130/183) were LBW

and among these, 13% (n=17/130) were very LBW (<1500 g). In this group of neonates, 22% were born SGA.

TABLE 3.1. Maternal and Neonatal Characteristics [1]

Maternal Characteristics[2]		
Characteristics	**Women Delivery Twins**	**Women Delivery Triplets/Quadruplets**
Gestational age (week)	35.6 ± 2.3 (n=64)[a]	32.4 ± 2.8 (n=19)[b]
Preterm birth, % (n)	56.3 (n=36/64)[a]	94.7 (n=18/19)[b]
Early term birth, % (n)	6.3 (n=4/64)[a]	26.3 (5/19)[b]
Cesarean section, % (n)	64.1 (n=41/64)[a]	94.7 (18/19)[b]
Discordant Growth, %		
>15% between siblings	20.3 (n=13/64)[a]	68.4 (n=13/19)[b]
>25% between siblings	6.3 (n=4/64)[a]	26.3 (n=5/19)[b]
Race		
African-American, % (n)	31.3 (n=20/64)[a]	5.3 (n=1/19)[b]
Caucasian, % (n)	67.2 (n=43/64)	84.2 (n=16/19)
Other, % (n)	1.6 (n=1/64)	10.5 (n=2/19)
Ethnicity		
Hispanic, % (n)	6.3 (n=4/64)	5.3 (n=1/19)
Non-Hispanic, % (n), %	93.8 (n=60/64)	94.7 (n=18/19)
Neonatal Characteristics[3]		
Characteristics	**Twins**	**Triplets/Quadruplets**

Birth weight (g)	2358.1 ± 61.8 (n=126)[a]	1860.7 ± 110.5 (n=57)[b]
<2500 g, % (n)	60.3 (n=76/126)[a]	94.7 (n=54/57)[b]
<1500 g, % (n)	3.2 (n=4/126)[a]	22.8 (n=13/57)[b]
SGA[4]	23.8 (n=30/126)	19.3 (n=11/57)

[1]Data are presented as the mean ± SD (Range) or the percentage, with the sample size in parentheses. Means of birth weight were calculated using a random effects model adjusting for the non-independence of data from siblings.

[2]Different superscripts in the table indicate significant differences in maternal characteristics between twins vs. triplets/quadruplets, (P<0.05, 2-tailed t test or chi square test).

[3]Different superscripts in the table indicate significant differences in neonatal characteristics between twins vs. triplet/quadruplets, (mixed model with random effect, p<0.05).

[2]SGA = small for gestational age

Neonatal anemia, and cord Fe status and inflammation at Birth

Neonatal anemia, Fe status and inflammatory markers are presented in **Table 3.2**. Anemia was found in 14% (n=22/153) of neonates at birth. No significant difference in the prevalence of anemia was found between the preterm and early-term neonates [16% (n=16/102) vs. 12% (n=6/51), P=0.3] and Hb was not significantly associated with GA at birth in this cohort (across the range of 25-38 weeks of gestation). The prevalence of anemia did not

significantly differ between LBW and normal-weight neonates [14% (n=15/110) vs. 16% (n=7/43), P=0.7]. Mean cord Hb in our study population was compared to normative cord Hb data obtained in 24,416 singletons (27) (**Figure 3.2).** Mean cord Hb concentrations in our neonates fell below the mean expected in singletons at all GA, and began to further diverge from the mean at ~31 weeks.

TABLE 3.2. Iron status at birth in 183 multiple birth neonates [1, 2]

	n	Preterm	n	Early-Term
Gestational age (week)	124	33.4 ± 0.3[a]	59	37.5 ± 0.07[b]
Hemoglobin (g/dL)	103	15.1 ± 0.3	51	15.3 ± 0.4
Anemia[3], %	17	17	6	12
Serum SF (µg/L)	93	96.4 [79.7, 116.7]	51	112.8 [85.8, 148.3]
Serum sTfR (mg/L)	93	5.8 [5.1, 6.5][a]	51	6.6 [5.9, 6.5][b]
Serum EPO (mIU/mL)	88	16.6 [13.2, 21.0]	50	18.8 [15.4, 22.9]
Serum Iron (mg/L)	91	4.1 [3.2, 5.2]	49	2.7 [2.2, 3.3]
Serum Hepcidin (ng/mL)	92	12.1 [9.2, 15.7][a]	50	17.0 [12.0, 24.2][b]
Hepcidin < 0.39 ng/mL, %	1	1	1	2
Serum IL-6 (pg/mL)	88	4.0 [3.3, 4.8]	50	3.7 [2.7, 5.1]
IL-6 < 0.156 pg/mL, %	0	0	0	0
CRP (ng/mL)	22	97.4 [80.7, 117.6]	19	102.7 [82.3, 128.0]
CRP < 78 ng/mL, %	62	74	31	62

[1] Data are presented as the mean ± SD for gestational age and Hb. Geometric mean [95% CI] for SF, sTfR, EPO, serum Fe, serum hepcidin, IL-6, and CRP.

Means were calculated using a random effects model adjusting for the non-independence of data from siblings.

[2] Different superscripts within rows indicate statistical differences between preterm and early-term neonates, (P<0.05,mixed model with random effects)

[3] Neonatal anemia was defined as a cord hemoglobin (Hb) < 13.0 g/dL.

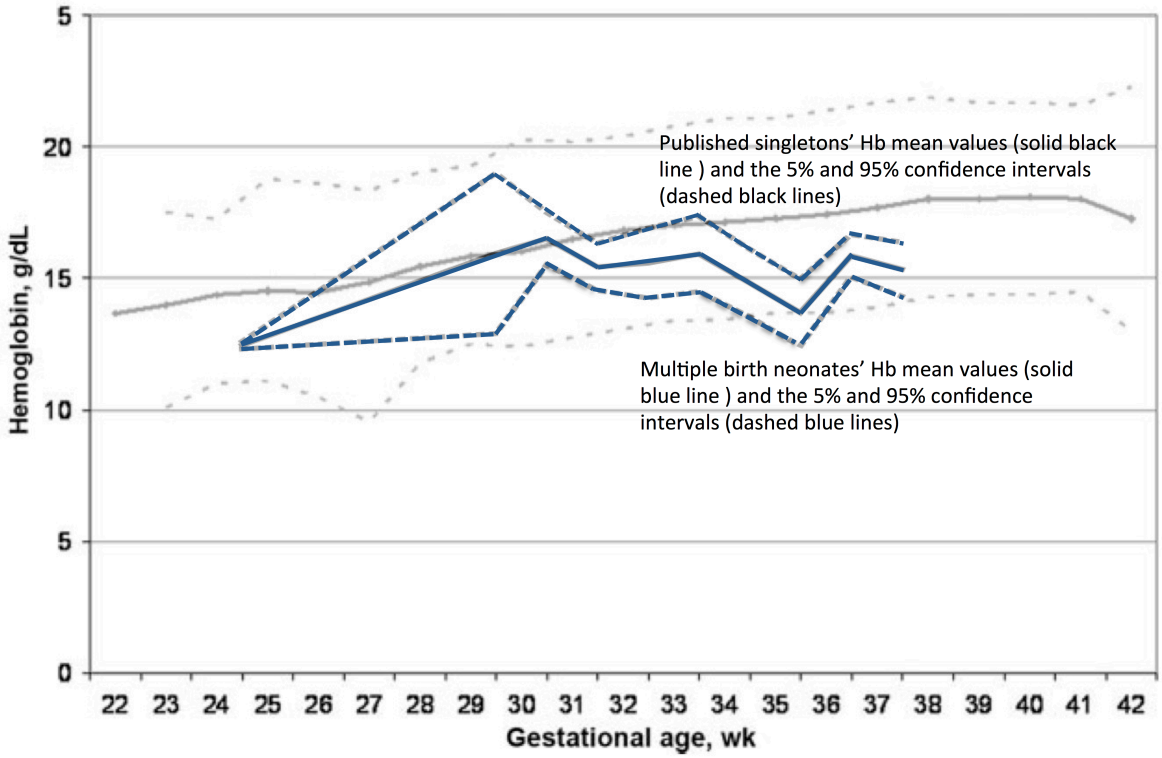

FIGURE 3.2 LEGEND

Figure 1. *Mean cord Hb of multiple birth neonates (solid blue line) was compared to published reference ranges in 24,416 singletons born between 22 to 42 weeks of gestation (solid black line) (24). The Hb curve (adjusted for the non-independence of the multiple siblings) in our population tended to fall*

below the normative curve, and a marked reduction was evident at 34 weeks of gestation. Figure 3.2 was adapted from Jopling et al (27).

Similar to findings for Hb, using a mixed model with random effects, no significant differences in cord SF, EPO, serum Fe, IL-6, or CRP were evident between the preterm and early-term neonates. In addition, GA had no significant association with cord SF, serum Fe, IL-6, or CRP across the GA at birth in this cohort. Using the same statistical method, cord sTfR (β=0.04, SE=0.02, P=0.02, n=144), EPO (β=0.10, SE=0.03, P=0.001, n=138), and hepcidin (β=0.11, SE=0.05, P=0.05, n=144) were significantly associated with GA at birth. However, preterm neonates (33.2 ± 2.4 weeks of gestation) had significantly lower cord hepcidin (14.8 ng/mL, n=92 vs. 20.8 ng/mL, n=50, P=0.02) compared to early-term neonates (37.5 ± 0.5 wks gestation) after controlling for non-independence of the multiple birth siblings.

To assess the degree to which greater birth weight might have influenced observed associations between GA and Fe status, all Fe status indicators were expressed on a per kg of body weight basis and potential associations with GA were reexamined. Cord sTfR/kg birth weight (β=-0.05, SE=0.02, P=0.002, n=144) and serum Fe/kg birth weight (β=-0.11, SE=0.02, P<0.0001, n=140) were negatively associated with GA at birth such that significantly higher concentrations of these indicators/kg birth weight were evident in neonates born at an earlier GA. All other Fe status indicators did not significantly vary across the window of 25-38 weeks when expressed on a per

kg basis.

Hepcidin was detectable at birth (37.5 ± 0.7 wks) in all but 2 neonates studied. Both of these neonates were non-anemic and they were born at 37 weeks or 35 weeks of gestation. Deficiencies of vitamin B-12 or folate were not evident in any of the neonates studied, and all neonates had detectable IL-6 at birth (25-38 wks) with values ranging from 0.46 - 40.24 pg/mL. In contrast, 69% of neonates (n=93/134) had undetectable cord CRP. In neonates with detectable cord CRP (n=41), a significant positive association was found between cord CRP and cord IL-6 (β=0.48, SE=0.10, P<0.0001, n=39).

Predictors of neonatal anemia at birth

Neonates born to women who were obese before entering pregnancy had more than a 4-fold greater odds of having anemia at birth compared to neonates born to normal-weight women (OR=4.1 [95% CI: 1.3, 15.6], P=0.02) using a mixed model with random effects and adjusting for birth weight and GWG). Using the same method, neonates born to women who smoked during pregnancy (n=12) had a nearly 5-fold greater odds of having anemia compared to neonates born to non-smokers (OR=4.5 [95% CI: 1.1 17.3], p=0.03). When the odds ratio examining the impact of smoking on anemia was evaluated after adjusting for birth weight and GWG, the impact of smoking during pregnancy on anemia was reduced and only approached significance (OR=4.2, [95% CI: 0.97, 16.6], p=0.07) **(Table 3.3)**. Prevalence of anemia did not significantly differ as a function of race (14% in Caucasian vs. 16% in

African-American neonates, p=0.6). Cord Hb was positively associated with Apgar scores at both 1 and 5 minutes (β=0.26, SE=0.09, P=0.005, n=154; β=0.48, SE=0.15, P=0.002, n=154, respectively) after adjusting for GA and birth weight.

Table 3.3 Maternal Determinants of Neonatal Anemia at Birth

	% Anemic at birth (n)	Unadjusted[1] OR [95% IC]	Adjusted[1,2] OR [95% IC]
Pre-Pregnancy BMI			
Obesity (n=51)	14% (n=12)	4.5 [1.4, 16.9]	4.1 [1.3, 15.6]
Normal weight (n=62)	6% (n=4)	Reference	Reference
Smoking during pregnancy			
Yes (n=10)	40% (n=4)	4.5 [1.1,17.3]	4.2 [0.97, 16.6]
No (n=139)	13% (n=18)	Reference	Reference

[1]Both unadjusted and adjusted OR were controlled for the non-independence of neonates born to the same mother using a mixed model with random effects

[2]Adjusted OR were adjusted for both GWG and neonatal birth weight

Correlations between cord Hb and other cord Fe indicators

Significant associations were found between Hb and all cord Fe status indicators except for serum Fe and EPO after correcting for multiple comparisons using a Bonferroni test (P<0.002). Cord SF, sTfR, EPO and

hepcidin were also significantly associated with each other **(Table 3.4)**. Cord sTfR exhibited the strongest correlation with Hb and alone captured 41% of the total variance in Hb, followed by hepcidin, which explained 37% of the total variance in Hb. A significant inverse association was evident between Hb and SF (β=-1.1, SE=0.23, p<0.0001, n=126), a finding that remained significant after adjusting for GA and birth weight. Anemia did not occur in neonates with SF under the 10th percentile (40.8 µg/L), but was present in 21% (n=3/14) of neonates whose SF was greater than the 90th percentile (250.7µg/L). Cord IL-6 and CRP did not significantly differ between neonates whose cord SF concentrations were either < 10th or >90th percentiles.

TABLE 3.4: Correlations between Iron Status Indicators in Cord Blood

	Log cord SF	**Log cord TfR**	**Log cord EPO**	**Log cord Serum Fe**	**Log cord Hepcidin**	**Log cord IL-6**
Cord Hb (g/dL)	β=-1.1 SE=0.23 **P<0.0001** n=126	β=2.6 SE=0.37 **P<0.0001** n=126	β=0.64 SE=0.24 P=0.009 n=121	β=0.46 SE=0.32 P=0.16 n=123	β=-0.83 SE=0.17 **P<0.0001** n=126	β=0.35 SE=0.22 P=0.12 n=122
Log Cord SF (µg/L)		β=-0.82 SE=0.14 **P<0.0001** n=144	β=-0.38 SE=0.08 **P<0.0001** n=138	β=0.18 SE=0.10 P=0.08 n=140	β=0.53 SE=0.05 **P<0.0001** n=144	β=0.05 SE=0.07 P=0.48 n=137
Log cord TfR (mg/L)			β=0.28 SE=0.04 **P<0.0001** n=138	β=0.006 SE=0.06 P=0.91 n=140	β=-0.23 SE=0.03 **P<0.0001** n=144	β=0.06 SE=0.04 P=0.17 n=137
Log cord EPO (mIU/mL)				β=0.03 SE=0.11 P=0.80 n=137	β=-0.35 SE=0.06 **P<0.0001** n=138	β=0.20 SE=0.07 P=0.009 n=134
Log Serum Fe					β=0.07	β=0.12

				SE=0.06 P=0.20 n=140	SE=0.06 P=0.054 n=136
Log cord Hepcidin (ng/mL)					β=0.04 SE=0.10 P=0.66 n=137

[1]*Bolded values denote associations that are significant at p<0.002 using a random-effects mixed model and applying a Bonferroni correction to correct for multiple comparisons.*

Correlations between Maternal and Cord Iron Status Indicators

Cord sTfR was significantly positively associated with maternal sTfR at 24.4 ± 5.4 wks (β=0.25, SE=0.09, p=0.01, n=128) and at delivery (34.6 ± 2.8 wks) (β=0.25, SE=0.08, p=0.002, n=120). At delivery, positive associations were also found between cord Hb and maternal Hb (β=0.37, SE=0.16, p=0.02, n=153) and hepcidin (β=0.22, SE=0.08, p=0.008, n=118). However, while cord and maternal hepcidin were correlated, only cord hepcidin was significantly correlated with umbilical cord Fe status indicators, including Hb (β=-0.83, SE=0.17, p<0.0001, n=126), SF (β=0.53, SE=0.05 p<0.0001, n=144), sTfR (β=-0.23, SE=0.03, p<0.0001, n=144), and EPO (β=-0.35, SE=0.06, p<0.0001, n=138). Associations between maternal hepcidin and cord Fe status only approached significance for cord SF (β=0.13, SE=0.06, p=0.06, n=118) and cord sTfR (β=-0.08, SE=0.04, p=0.054, n=118).

3.5 DISCUSSION

To our knowledge, this is the largest study to explore Fe status in neonates born to women with multiple gestations and to evaluate temporal changes in neonatal Fe status in neonates born between 25-38 weeks of gestation. Of note, anemia was present in 14% of neonates studied. Neonates born to obese women and women who smoked during pregnancy had significantly greater odds of anemia at birth. Hepcidin was detectable at birth in nearly all neonates born between 25-38 weeks of gestation, and cord hepcidin, but not maternal hepcidin, was significantly associated with multiple umbilical cord Fe status indicators.

At birth, anemia was evident in 14% of neonates studied (17). There are no national data on anemia prevalence in singleton newborns for direct comparison as NHANES studies do not enroll those under 1 year of age (28). Compared to reference cord Hb data from 24,416 singleton neonates (22-42 weeks of gestation) (27), our Hb curve diverged from the reference curve at ~31 weeks of gestation and a marked reduction was evident by 34 weeks of gestation. This likely reflects an inability of placental transport to keep pace with the rapid fetal growth of multiple fetuses (29, 30), as the fetal growth trajectory in multiple pregnancies is also known to diverge from the singleton reference curve between 30-32 weeks of gestation (31, 32).

The American Academy of Pediatrics highlighted PTB as a risk factor for neonatal anemia (17). In our population, preterm neonates did not have an increased risk of anemia compared to early term neonates, however because

we had a power of only 79%, we were not sufficiently powered to evaluate possible differences in anemia between these two groups. Moreover, a longer duration *in utero* did not result in a significant accumulation of Fe stores or Hb, although body Fe continued to accumulate as each Fe indicator remained constant across gestation when expressed on a per kg of body weight basis. This finding differs from data in preterm singletons where many studies have found a higher risk of anemia and lower Fe status in preterm singleton newborns (33-38).

Risk of anemia was 4-fold higher in neonates born to women who were obese before pregnancy. The adverse effect of maternal ppBMI on neonatal anemia was evident only in women whose ppBMI exceeded 30 kg/m^2; this difference was not observed among women who were overweight (25-30 kg/m^2) at entry into pregnancy. Data on maternal obesity and neonatal Fe status are limited and often contradictory. We previously found that adolescents (n=230) in higher ppBMI categories gave birth to neonates with significantly higher cord Hb (39), but these unexpected findings may be due to biological immaturity of the adolescent as animal models have suggested that maternal-fetal nutrient partitioning differs among adolescents compared to adults (40, 41). In a primarily adult population, studies in 316 U.S. and 1613 Chinese women found adverse effects of maternal obesity on neonatal Fe stores (42, 43). Further studies are needed to understand the impact of maternal obesity on neonatal Fe status in some, but not all, populations.

Risk of neonatal anemia was higher in multiple birth neonates whose

mothers smoked during pregnancy. While studies have found cigarette smoking to be negatively associated with neonatal ferritin, transferrin, and total body Fe (44, 45), few data are available on maternal cigarette smoking and risk of neonatal anemia. Because cigarette smoking is a modifiable risk factor, its adverse effects on neonatal anemia in women carrying multiples should be highlighted especially as it is also known to be a risk factor for PTB, LBW, fetal growth restriction and infant death (46), outcomes which already occur at increased rates in multiple birth neonates.

To date, data on associations between cord and maternal hepcidin have been mixed (47-49). In our study, cord hepcidin was significantly associated with maternal hepcidin at delivery, but only cord hepcidin was correlated with cord Fe status and this hormone alone captured nearly 37% of the total variance in cord Hb. These data suggest that the fetus independently regulates its Fe status, a finding consistent with other data from full-term singleton neonates (47, 48).

In these multiple birth neonates, most cord Fe indicators evaluated exhibited the expected associations with cord Hb based on existing data on Fe indicators in term or preterm singletons (48, 50). Among the indicators evaluated, cord sTfR was the strongest determinant of cord Hb, and alone captured 41% of the total variance in cord Hb. Given the association between cord sTfR and maternal sTfR at mid-gestation, maternal sTfR may be a useful predictor of low cord Hb at birth. Unlike observations made in older infants and adults, a significant inverse association was found between cord SF and Hb.

Nearly 1/4 of neonates with the highest SF had anemia whereas anemia was not found in infants below the 10th percentile of the SF distribution. This unexpected observation was not driven by inflammation as assessed using IL-6 or CRP. A similar inverse SF and hepcidin association was noted in term singletons born to adolescents (48). Because SF is utilized as a common index of Fe sufficiency in newborns, further data on this unexpected association are needed.

This study has several limitations. There are few normative data on Fe status in neonates to use for comparison purposes. Moreover, multiple assays are available for the Fe indicators evaluated and most are not standardized, limiting comparisons between studies. Secondly, because there are currently no validated human assays for erythroferrone, we were unable to evaluate this hormone which is known to be stimulated by EPO and mediate hepcidin suppression (51). While we studied a relatively large cohort of multiple birth neonates, our sample size was reduced when analyses were stratified, lowering power to detect significant associations. Finally, although IL-6 and CRP are two standardly utilized inflammatory markers, the presence of inflammation could not be ruled out and normative data on these markers are limited.

In conclusion, neonates born to women carrying multiple fetuses had a greater risk of anemia than has been reported among term singletons. Hepcidin was present at birth in nearly all neonates studied was significantly associated with cord Fe status highlighting the ability of the fetus to regulate its

Fe status in response to its intrauterine Fe environment. Increased time in utero from 25-38 weeks did not reduce the risk of anemia or lead to an increase in Fe status for the majority of Fe status indicators evaluated. Multiple birth neonates born to women who were obese prior to pregnancy, or to those that smoked during pregnancy had a higher risk of anemia. Additional screening practices for Fe status at birth are warranted in these groups. Given the higher risk of anemia noted, further studies are needed to identify maternal interventions that best support fetal Fe requirements in this higher risk neonatal population.

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CHAPTER 4

INTER- AND INTRA-UTERINE VARIANCE IN HEPCIDIN AND IRON STATUS IN MULTIPLE BIRTH NEONATES

To be submitted to The FASEB Journal in 2017

Yuan Ru, Eva K. Pressman, Ronnie Guillet, Philip J. Katzman, Vermeylen, F, Kimberly O. O'Brien. Inter- and intra-uterine variance in hepcidin and iron status in multiple birth neonates

4.1 ABSTRACT

Background: Hepcidin is a systemic regulator of iron (Fe) homeostasis. Little is known about the relative role of maternal versus neonatal hepcidin on neonatal Fe homeostasis. Multiple births are a unique *in vivo* human model to address this question.

Objective: We evaluated inter- and intrauterine variances in neonatal Fe, vitamin B-12, and folate status, and inflammatory markers in a large cohort of multiple birth neonates to assess the relative impact of neonatal or maternal hepcidin on neonatal Fe status and to identify significant determinants of neonatal Fe status.

Design: A total of 183 multiple neonates were recruited at birth. Umbilical cord blood samples were obtained to assess hemoglobin, serum ferritin (SF), soluble transferrin receptor (sTfR), hepcidin, serum iron, erythropoietin, serum folate, vitamin B-12, C-reactive protein (CRP) and interleukin-6.

Results: Cord hemoglobin (p=0.34), serum Fe (p=0.36), hepcidin (p=0.39), and CRP (p=0.28) had greater intrauterine variance than interuterine variance. In contrast, folate (p=0.74) and vitamin B-12 (p=0.79) were more variable between unrelated neonates than between siblings. Cord hepcidin alone captured 63.8%, 45.2%, 44.4%, and 31.3% of the intra-uterine variances of cord hemoglobin, SF, sTfR, and erythropoietin, respectively. Significantly greater differences in cord SF, sTfR, hepcidin, and EPO were found between di- and trichorionic siblings than between monochorionic siblings. Pre-pregnancy body mass index and cigarette smoking during pregnancy

explained 20.5% and 20.6% of the inter-uterine variance in cord Hb, respectively. Maternal Hb and sTfR at delivery explained 23.4% and 25.3% of the inter-uterine variance in cord Hb and sTfR, respectively.

Conclusion: Fetally derived hepcidin appears to be capable regulating fetal Fe status. Cord Fe status indicators known to be regulated by hepcidin exhibited more variability between siblings than between unrelated neonates. For folate and vitamin B-12 that not known to be regulated by fetally derived hormone, a shared uterine cavity had the largest impact on regulating their homeostasis. Using a multiple births model might provide a unique way to examine factors that contribute to placental nutrient transport and fetal nutrient partitioning.

4.2 INTRODUCTION

Hepcidin is a hormone that systemically regulates iron (Fe) homeostasis. It limits the release of Fe into the circulation by internalizing ferroportin and has also been found to down-regulate the expression of the cellular Fe import protein divalent metal transporter 1 (DMT-1) in the enterocyte (1-3). Of interest, the fetus begins to produce hepcidin early in the 1st trimester as determined by the presence of pro-hepcidin (precursor of hepcidin) in fetal blood at 8-11 weeks of gestation (4). Studies on cord blood hepcidin concentrations among multiple birth neonates have also found detectable hepcidin in 98% of neonates born between 25-38 weeks of gestation (5). Two recent studies evaluating Fe status between maternal and neonatal dyads have found that cord hepcidin, but not maternal hepcidin, is significantly associated with neonatal Fe status at birth (5-7), indicating that this fetally-derived hormone may independently regulate fetal Fe status.

Animal models provide opportunities to fully delineate the role of fetal hepcidin across gestation. Transgenic mice designed to overexpress hepcidin developed Fe deficiency and anemia i*n utero* and were born with severe Fe deficiency anemia (8, 9). Other transgenic murine models have found that fetal mice with repressed hepatic expression exhibited greater placental expression of Fe transporter proteins and accrued more hepatic Fe than wild type mice (10), data that further highlights the regulatory capacity of fetal hepcidin.

The placenta is exposed to both maternal and neonatal hepcidin. Using stable Fe isotopes, human data have found significant associations between

maternal hepcidin and placental transfer of non-heme Fe, suggesting that maternal hepcidin also plays a regulatory role in Fe transport across the placenta (11). At present, it is difficult to isolate the relative role of maternal versus fetal hepcidin on fetal Fe homeostasis in humans. One possible *in vivo* human model that can be used to address this question is multiple births, as this provides an opportunity to evaluate the variance in Fe status at birth between siblings who shared the same intrauterine environment and that were exposed to the same maternal hepcidin concentrations across gestation.

To date, few data are available on intra-uterine variance in neonatal Fe status. Animal studies have been interested in intra-uterine variation in birth weight because it is economically important for the meat industry due to the positive correlation with pre-weaning mortality, poor immunity, and low nutritional status after birth (12, 13). Factors that have been found to influence the intra-uterine birth weight variation in animal models include placentation, hormone concentrations, and efficiency of placental transport of nutrients (14-16).

To further explore the ability of fetally derived hepcidin to regulate Fe status, the goal of this study was to evaluate inter- and intra-uterine variances in neonatal Fe, vitamin B-12, folate and inflammatory markers in a large cohort of twins, triplets and quadruplets. The goals of the study were to assess the relative impact of neonatal versus maternal hepcidin on neonatal Fe status at birth and to identify other significant determinants of inter- and intra-uterine variance in neonatal Fe status.

4.3 SUBJECTS AND METHODS

Participants

The study population was comprised of a total of 186 neonates including 64 sets of twins (n=128), 18 sets of triplets (n=54), and 1 set of quadruplets (n=4). All infants were born between 2011 and 2014 at Strong Memorial Hospital or Highland Hospital at the University of Rochester, in Rochester, NY. Neonates in the study were born to 83 healthy adult women who did not have HIV infection, pre-existing diabetes mellitus, hemoglobinopathies, or other medical problems known to affect Fe homeostasis at the time of enrollment. All women had received standard prenatal supplementation providing from 27-90 mg of Fe per day as part of their routine prenatal care. Data on Fe status in these 83 women have been reported and data on their neonates are under review for publication (17, 5). This study was approved by the Institutional Review Boards of the University of Rochester and Cornell University.

Data on gestational age (GA) at birth, preterm birth, and low birth weight (LBW) were determined following standard methods (17). Neonates were categorized as small-for-gestational age (SGA) if their birth weight was <10th percentile using normative growth curves (18). Discordant growth (> 15% difference between siblings) was defined following published procedures (19). Neonatal anthropometric data (birth weight, length, and head circumference) were reviewed and abstracted by study personnel from infant medical charts. All placentas were sent to the Strong Memorial Pathology Laboratory to be

assessed by placental pathologists who evaluated chorionicity (the number of placental disks), and amnionicity (the number of amniotic sacs) for each set of multiples as mono-, di-, tri-, or quadra- for each of the chorionic and amniotic variables following established criteria (20).

Neonatal Blood Collection and Biochemical Analyses

Umbilical cord blood (15 mL) was collected at delivery and cord hemoglobin (Hb) was measured at the University of Rochester Strong Memorial Hospital clinical laboratory using a Cell-Dyn 4000 hematology analyzer (Santa Clara, CA) following Clinical Laboratory Improvement Amendments (CLIA) methodology. Neonatal anemia was defined using standard criteria, as a cord Hb concentration < 13 g/dL and data on Fe status in this cohort have been previously published (21).

All neonatal Fe status indicators were measured at Cornell University, Ithaca, NY. Serum ferritin (SF) and soluble transferrin receptor (sTfR) were analyzed using ELISAs (Ramco Laboratories, Inc. Stafford, TX). Cord hepcidin was analyzed using an enzyme immunoassay kit (Bachem, CA), which detected concentrations > 0.39 ng/mL (20). Values under this concentration were assigned a value of 0.195 ng/mL for analysis purposes. Cord serum Fe was measured using graphite furnace atomic absorption spectrophotometry (Perkin Elmer AAnalyst 800). Cord serum C-reactive protein (CRP) was measured using a Quantikine ELISA kit (R&D System, MN). Serum IL-6 concentrations were assessed using a high sensitivity Quantikine ELISA kit

(R&D System, MN). Concentrations of CRP under 0.078 mg/L and IL-6 concentrations under 0.156 ng/mL were below each respective assay's limit of detection and were assigned values of 0.039 mg/L and 0.078 ng/mL, respectively for analysis purposes. Erythropoietin (EPO), folate, and vitamin B-12 were analyzed using a Siemens Immulite® 2000 immunoassay system. Cord folate and vitamin B-12 concentrations under 6.8 nmol/L or 148 pmol/L, respectively, were classified as insufficient. Maternal Fe and inflammation indicators were analyzed using the same procedures above as previously detailed (20).

Statistical Analysis

All continuous variables were tested for normality of distributions using the Shapiro-Wilk test and if they were not normally distributed, they were logarithmic transformed, and geometric means adjusting for non-independent variables were reported. Interuterine variance was defined as the observed variance in each neonatal Fe status indicator between unrelated neonates (i.e. between different uterine cavities); intrauterine variance was defined as the variance observed in neonatal Fe status indicators within the same uterine cavity (i.e. between siblings). The intraclass correlation coefficient (ICC) was utilized to evaluate the between- (interuterine variance) and within-cluster variances (intrauterine variance). The proportion of the total variance that was explained by interuterine variance, stated as p was calculated as the between-cluster variance divided by the sum of the within- and between-cluster

variances. Using this approach, for any given Fe status indicator or neonatal birth weight, the resulting p value could range from 0 to 1, where values close to 0 are indicative of greater intra-uterine variance (i.e. more variability between siblings) and values close to 1 are indicative of lower intrauterine variance (i.e. less variability between siblings than observed between unrelated neonates) (**See Figure 4.1**). The intrauterine difference for each neonatal Fe status indicator was then determined by subtracting the smallest concentration from the largest observed concentration of each indicator within a uterine cavity. To identify significant determinants of inter- and intrauterine variances, a generalized mixed model was used to test the significance of the difference in coefficients between full models (with determinants) and reduced models (without determinants). Our sample size of 183 neonates was sufficient to detect correlations on the order of 0.15-0.20, with an alpha level of 0.05. Statistical tests were considered significant at $p < 0.05$. All statistical analyses were performed using JMP 12.0 (SAS Institute Inc, Cary, NC).

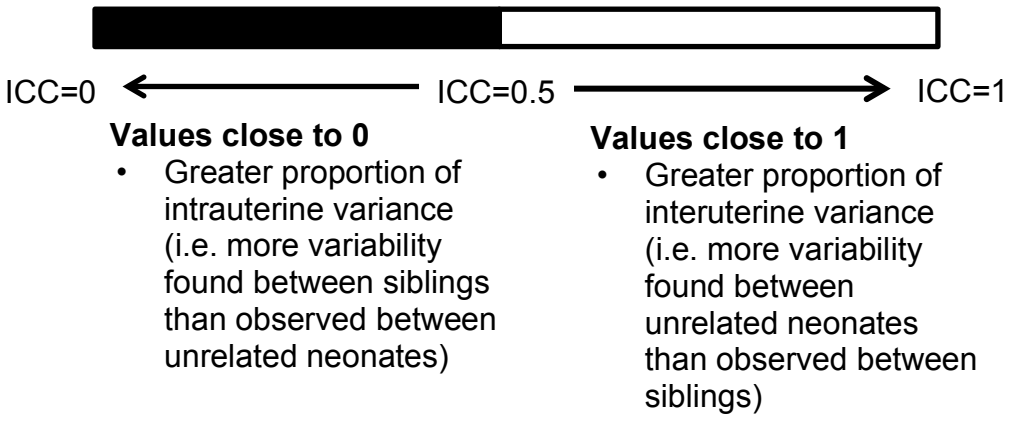

Figure 4.1 Interpretations of Intraclass Coefficients

4.4 RESULTS

Neonatal Characteristics

Among the 186 neonates, there were 3 cases of neonatal mortality, which resulted in a final study population of 183 neonates. Maternal and neonatal characteristics are presented in **Table 4.1**. In this group of neonates, there were 64 sets of twins, 18 sets of triplets and 1 set of quadruplets. Among the 64 sets of twins, 73% (n=47) were dichorionic and diamnionic, 23% (n=15) were monochorionic and diamnionic, and 3% (n=2) were monochorionic and monoamniotic. Among the 18 sets of triplets, 83% (n=15) were trichorionic and triamniotic, 11% (n=2) were dichorionic and triamniotic, and 5% (n=1) were monochorionic and triamniotic. The one set of quadruplets was dichorionic and tetraamniotic. Data on Fe status of this neonatal cohort have been previously reported (5).

TABLE 4.1. Characteristics of multiple birth neonates[1]

Characteristics	Mean ± SD or % (n)
Maternal Characteristics, n=83	
Gestational age at birth (weeks)	34.8 ± 2.7
Preterm (< 37 wks), % (n)	65 (54)
Early-Term, (37-38 wks) % (n)	35 (29)
Mode of Delivery	

Vaginal delivery, % (n)	29 (24)
Cesarean section, % (n)	71 (59)
Race	
African-American, % (n)	25 (21)
Caucasian, % (n)	71 (59)
Other, % (n)	4 (21)
Ethnicity	
Hispanic, % (n)	6 (5)
Non-Hispanic, % (n)	94 (78)
Discordant Growth, %	
> 15% between siblings	34.4 (63)
> 25% between siblings	11.5 (21)
Neonatal characteristics, n=183	
Birth weight (g)	2206.5 ± 571.5
<2500 g, % (n)	71 (130)
<1500 g, % (n)	9 (17)
SGA[3]	22.4 (41)
Apgar score (1 min)	7.1 ± 2.2

[1]Data are presented as mean ± SD, or percentage. Means of neonatal birth weight and Apgar score were calculated using a random effects model adjusting for the non-independence of siblings.

[2]SGA = small for gestational age

Interuterine and intrauterine variances

The ICC values (p) for each neonatal Fe indicator and birth weight are reported in **Table 4.2**. Cord Hb (p=0.34), serum Fe (p=0.36), hepcidin (p=0.39), and CRP (p=0.28) had greater intrauterine variance than interuterine variance. Values close to 0.5 were found for cord SF (p=0.44), EPO (p=0.46), sTfR (p=0.47), and IL-6 (p=0.52), suggesting approximately equal intra- and interuterine variances. Unlike the findings for several of the Fe status indicators and CRP, higher interuterine variances were evident for cord vitamin B-12 (p=0.74), and folate (p=0.79). Neonatal birth weight (p=0.79) also had greater variability between unrelated neonates than between siblings.

TABLE 4.2. ICC Values of Birth Weight and Cord Fe Status Indicators[1]

Fe Status Indicators	N	ICC[2]	95% CI for ICC
Cord CRP	134	0.28	0.03-0.41
Cord Hb	153	0.34	0.12-0.46
Cord Serum Fe	140	0.36	0.13-0.47
Cord Hepcidin	144	0.39	0.17-0.48
Cord SF	144	0.44	0.22-0.53
Cord EPO	138	0.46	0.26-0.53
Cord sTfR	144	0.47	0.28-0.55
Cord IL-6	138	0.52	0.32-0.59
Cord B-12	116	0.74	0.56-0.75
Cord Folate	128	0.79	0.63-0.81

Birth Weight	183	0.79	0.66-0.79

[1]Data are presented as the sample size, ICC values, and 95% CI for ICC

[2]ICC = Intraclass correlation coefficients

Effects of chorionicity and amnionicity on intra-uterine variance

Potential differences in Fe status indicators between siblings were also evaluated as a function of chorionicity and amnionicity. As expected, significantly greater differences in cord SF (21.8, n=14 vs. 44.8, n=48; P=0.03), sTfR (0.87, n=14 vs. 1.7, n=48; P=0.03), hepcidin (1.7, n=14 vs. 7.7, n=48, P=0.0003), and EPO (3.6, n=12 vs. 8.6, n=46; P=0.03) were found in the group of di- and trichorionic siblings in comparison to that observed among the monochorionic siblings who shared the same placenta and vascular supply.

Unlike chorionicity, no significant differences in Fe status indicators were found between diamniotic and triamniotic siblings. Since there was only one set of monoamniotic twins and one set of tetraamniotic quadruplets who had complete data on Fe status in this study cohort, we were unable to make any statistical comparisons between neonates in these groups.

Effects of cord hepcidin on inter- and intrauterine variances

Cord hepcidin but not maternal hepcidin was previously reported to be significantly associated with multiple cord Fe indicators (5). The degree to which cord hepcidin contributed to the observed intra- and/or interuterine

variance in neonatal Fe status indicators was explored by comparing the difference in coefficients obtained from the full models (with hepcidin) compared to those found in the reduced models (without hepcidin). Using this approach, cord hepcidin was found to capture 63.8%, 45.2%, 44.4%, and 31.3% of the intrauterine variances of cord Hb, SF, sTfR, and EPO, respectively **(Table 4.3)**. Neonatal cord hepcidin concentration also explained nearly 40% of the observed interuterine variance in cord SF. No significant effect was found between maternal hepcidin at delivery or at mid-gestation and intrauterine variance in any of Fe status indicators examined. While hepcidin is known to also function as an acute phase protein, cord IL-6 had no significant effect on either inter- or intrauterine variance in cord hepcidin and cord IL-6 and hepcidin concentrations were not significantly associated with one another in this group of neonates as a whole. Cord CRP is another indicator of inflammation and similar analyses on this inflammatory marker found that CRP captured 4.7% of the interuterine variance in hepcidin but it also had no significant effect on intrauterine hepcidin variance. Neonatal sex and race had no significant effects on inter- or intrauterine variance of the cord Fe status indicators evaluated.

Table 4.3 Assessment of effect of cord hepcidin on other cord Fe indicators

Determinant	Values on intra-uterine variance coefficients			% of intra-uterine variance explained by cord hepcidin
	Total effect (95% CI)	Direct effect (95% CI)	Indirect effect[1] (95% CI)	
Cord hepcidin				
Cord Hb	4.7	1.7	3.0	63.8
Cord SF	0.31	0.17	0.14	45.2
Cord sTfR	0.09	0.05	0.04	44.4
Cord EPO	0.32	0.22	0.10	31.3

[1] Indirect effect = total effect (cord Fe indicators and cord hepcidin) – direct effect (cord Fe indicators alone); the proportion of intra-uterine variance explained by cord hepcidin was calculated as the indirect effect divided by total effect.

[2] Controlled for neonatal birth weight.

The possible impact of maternal Fe status and maternal characteristics on interuterine variance in neonatal Fe status indicators was also examined. Maternal pre-pregnancy body mass index (BMI) captured 20.5% of the inter-uterine variance in cord Hb, and maternal self-report of maternal cigarette smoking during pregnancy captured 20.6% of the interuterine variance in cord Hb. Maternal Hb at delivery also explained 23.4% of the interuterine variance in cord Hb. Maternal sTfR concentrations during pregnancy or at delivery

captured 6.9% and 25.3% of the interuterine variance in cord sTfR, concentrations, respectively. Maternal hepcidin concentration during pregnancy captured 8% and 20% of interuterine variance in cord EPO and serum Fe, respectively.

4.5 DISCUSSION

To date, the degree to which the fetus controls placental Fe trafficking has been difficult to determine in human studies. By exploring the variability in Fe status between twins, triplets, and quadruplets sharing the same uterus and being exposed to the same maternal nutritional and hormonal environment, we were able to assess the degree to which neonatal Fe status varied between siblings and the degree to which variable neonatal hepcidin concentrations contributed to observed differences in Fe status between siblings at birth.

Discordant growth is common among multiple birth neonates and prior data in a group of 183 multiple birth neonates found that 34% of neonates in this group exhibited at least a 15% difference in birth weight between siblings (17). Several other human studies on discordant twin growth have found that placentation and blood supply to the fetus impacts fetal growth (22-24). In this study, we found that 21% of the total variance in birth weight was explained by intrauterine factors. The observed intrauterine variance in this group of neonates is similar to the within litter variation in birth weight of 0.26-0.30 that was reported in a study of neonatal piglets from litters that were comprised of

≤11 to ≥ 16 litters (25).

Hepcidin is a fetally derived hormone that the fetus begins to produce within the first 90 days of life (4). We found that cord hepcidin concentrations at birth were more variable between siblings than between unrelated neonates. This fetally derived regulatory hormone explained the largest fraction of observed variability in cord Hb, SF, sTfR, and EPO between siblings. Previous studies that measured both maternal and neonatal hepcidin have also found cord hepcidin to be independently associated with neonatal Fe status indicators at birth (5-7). Few studies have identified factors that impact fetal production of this hormone *in utero* in humans due to the challenges inherent in obtaining fetal blood samples. Using transgenic murine models, the regulatory pathways that stimulate or inhibit hepcidin production (i.e. bone morphogenetic protein 6, BMP6, and matripatase-2) (9, 26-28) have been identified and continue to evolve. Additional research is needed to investigate these regulatory pathways across gestation and at birth in humans.

Cord serum Fe was the only Fe status indicator measured that was found to exhibit greater variability between siblings than between unrelated neonates, but the interuterine variability observed for this indicator was not significantly impacted by neonatal hepcidin. Cord serum Fe and hepcidin were not correlated with one another in this group of neonates (5) nor were these two indicators significantly related to one another in a cohort of neonates born to pregnant adolescents that was studied using similar methods (7). Serum Fe is not an optimal Fe indicator of Fe status as it only reflects the amount of

circulating Fe in cord blood and is not a sensitive measure of iron deficiency due to its high degree of daily fluctuation.

In contrast to the greater variability observed in several Fe status indicators between siblings, vitamin B-12 and folate were more variable between unrelated neonates than between siblings, suggesting that the shared maternal and intrauterine environment had a large impact on the neonatal folate and vitamin B-12 endowment of multiple siblings at birth. Unlike the regulatory role of fetal hepcidin in fetal Fe homeostasis, there are no known fetally derived hormones that are responsible for the regulation of B-12 and folate homeostasis. Our findings indicate that neonatal status of these nutrients at birth is largely driven by the shared maternal environment. Our finding is supported by other studies that have reported significant associations between maternal and neonatal vitamin B-12 and/or folate status (29-30).

Chorionicity is another factor that would be predicted to impact fetal nutritional supply and partitioning in multiple birth neonates. Di- or trichorionic siblings each have their own discrete placenta, whereas monochorionic siblings share a placenta and a placental vascular network (24). The situation becomes more challenging in higher order multiple birth neonates, as there are multiple possible combinations of chorionicity within a uterine cavity. In this group of multiple birth neonates, as would be expected we found that monochorionic siblings exhibited significantly less variability in the Fe status indicators evaluated compared to the corresponding variability observed

among the di- or trichorionic siblings at birth.

We previously found that both maternal ppBMI and cigarette smoking were significant risk factors for neonatal anemia in this same study population (5). In the current study these two maternal factors also exhibited a substantial impact on the variability in Fe status observed between unrelated neonates. Both of these maternal risk factors captured a significant amount of the observed interuterine variance in cord Hb concentrations. Our previous study found significant associations between maternal and cord Hb and sTfR (5), but in the current analysis maternal TfR and Hb concentrations only impacted the variability in cord Fe status observed between unrelated neonates.

Several limitations are evident in this study. Individual placental weights could not be obtained or estimated for fused di-/trichorionic placentas. This influenced our ability to evaluate outcomes as a function of placental weight. As our study approach is unique, there are no normative data on intra-uterine variability in Fe status that can be utilized for comparison purposes. It is possible that some aspects of this regulatory process may only become evident when there is a need to supply Fe to multiple fetuses and the differences observed in this model may not be evident when Fe availability is not limited.

In conclusion, adequate Fe supply to the fetus across gestation is essential to support Hb demands and other Fe-dependent functions. A substantial amount of variability in Fe status and Hb concentrations was evident at birth between siblings sharing the same uterine environment. Using

this unique human model, we found that cord hepcidin concentrations explained the largest amount of the observed variability in several neonatal Fe status indicators. Maternal hepcidin did not impact intrauterine variance in Fe status indicators between siblings. Evaluation of interuterine and intrauterine variance of Fe status among multiple birth siblings may provide a unique way to examine factors that contribute to nutrient transport and partitioning. Further research is needed to address the control of maternal versus fetal hepcidin on placental Fe trafficking, and to investigate regulatory mechanisms of fetal hepcidin production that may have clinical and intervention implications for the acquisition of optimal fetal Fe stores across gestation.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

5.1 SUMMARY

This doctoral dissertation has presented normative data on 1) the degree of anemia and ID in women carrying multiple fetuses and in their neonates at birth; 2) it evaluated temporal changes in maternal Fe status across pregnancy; and 3) it identified significant determinants of maternal and neonatal anemia in a high-risk obstetric cohort. This work also utilized a unique approach to evaluate interuterine and intrauterine variances in neonatal Fe status. These three specific aims have been individually detailed and discussed in Chapters Two through Four. Below is a brief summary of the main findings from each specific aim, followed by the implications of this research as a whole, and suggestions for future research.

The first specific aim of this research was to characterize longitudinal changes in maternal Fe status across pregnancy and to identify significant determinants of maternal ID and anemia. A total 83 women (30.3 ± 5.1 y) carrying twins, triplets, and quadruplets were recruited in this study. Maternal blood samples were collected during pregnancy and/or at delivery to assess maternal Fe status indicators, serum folate and vitamin B-12, and inflammatory markers. Women carrying multiple fetuses were found to have a

higher prevalence of anemia and ID than expected based on normative data from women carrying singletons. The prevalence of anemia increased significantly from the first to the third trimester. Nearly half of the study population was anemic during the third trimester, a prevalence that was 4-fold higher than that expected using national data in women carrying singletons. Women who were anemic in the second trimester had a nearly 2-fold increased risk of anemia at delivery. Like findings on maternal anemia, maternal tissue ID also increased significantly from pregnancy to delivery. Compared to 1999-2006 NHANES data, the prevalence of depleted Fe stores in women carrying multiple fetuses was significantly higher than expected during the first and second trimesters based on normative national data from women carrying singletons. Maternal EPO was found to be the strongest predictor of maternal anemia at delivery, given the significant associations found between EPO during pregnancy and Hb at delivery. This finding suggested that screening for elevated EPO at early gestation might help to identify women who are at high risk of developing anemia at delivery. These data also speak to the need for greater anemia screening and for increased iron supplementation in this population.

The second research aim was to characterize neonatal Fe status at birth and identify significant determinants of neonatal anemia at birth in neonates born to women carrying multiple fetuses. A group of 186 neonates (including 64 sets of twins, 18 sets of triplets and 1set of quadruplets) were enrolled in the study. In this study population, nearly 70% of neonates were

born prematurely and all others were born early term (<38 weeks of gestation). Anemia was present in 14% of neonates studied. Compared to the published reference ranges of the mean cord Hb in singletons born between 22-42 weeks of gestation, mean cord Hb in our multiple birth neonates fell below the normative curve, and a marked reduction was evident at 34 weeks of gestation. This finding is similar to data on fetal growth in multiple birth neonates, which has shown fetal growth to decline between 30-32 weeks of gestation likely due to a reduced capacity of placental transport to meet fetal demands (1, 2). In this study population, preterm neonates did not have a higher risk of anemia compared to early term neonates. Similar to findings for Hb, no significant differences in cord SF, EPO and serum Fe were found between preterm and early-term neonates. These Fe status indicators remained constant across late gestation (25-38 weeks) when expressed on a per kg basis. This result indicates that a longer duration i*n utero* did not result a significant accumulation of stored Hb or Fe. This finding differs from what has been reported among singletons. This observation may indicate that the maternal Fe supply or placental Fe transport capacity is not sufficient to keep pace with the Fe demands of multiple fetuses over late gestation. Since pre-pregnancy obesity and cigarette smoking are two modifiable risk factors, their adverse effects on neonatal anemia in women carrying multiples should be highlighted. Additional screening practices for Fe status at birth are warranted in neonates born to women who were obese prior to pregnancy or to those who smoked during pregnancy in order to prevent neonatal anemia at birth.

The final research aim was to evaluate inter- and intrauterine variances in neonatal Fe, vitamin B-12 and folate status, and inflammatory markers in multiple birth neonates (n=183) and to identify other significant determinants of inter- and intrauterine variance of neonatal Fe status indicators. In this study, we found that fetally derived hepcidin was a significant determinant of fetal Fe status. Cord hepcidin exhibited more variability between siblings than between unrelated neonates, and hepcidin alone explained the largest fraction of observed variability in multiple cord Fe status including Hb, SF, sTfR, and EPO between siblings. In contrast, vitamin B-12 and folate, two nutrients that are not known to be regulated by fetally derived hormone were more variable between unrelated neonates, indicating that a shared uterine cavity has the largest impact on folate and vitamin B12 status. As expected, neonates who shared a placenta had significantly lower variance in Fe indicators compared to those who had their own placenta. Since this is the first study to characterize inter- vs. intrauterine variance for a full panel of neonatal Fe indicators, it might provide a unique approach to identify factors that contribute to placental nutrient transport and fetal nutrient partitioning.

5.3 LIMITATIONS

This research has several limitations. Human participants are always subject to loss to follow-up and may drop out of the study due to unexpected complications. In this study, in some instances maternal blood samples were not collected during pregnancy (n=10) or at delivery (n=22) due either to

emergency conditions; the study personnel were not notified or not available for blood collection; or the participant refused the blood draw. Umbilical cord blood samples were not collected (n=39) due to emergency delivery conditions. The study sample size was reduced when analyses were stratified, lowering power to detect significant associations.

There are few normative data on maternal and neonatal anemia, ID, or IDA to use for comparison purposes when interpreting findings generated. Moreover, most assays that are commercially available are not standardized, which also limits comparisons between studies. For the maternal cohort, we did not recruit a reference population of women carrying singletons for comparison purposes. The dietary data obtained were often incomplete and were not obtained using gold-standard approaches, limiting our ability to utilize our dietary data. In addition, maternal supplement compliance data was self-reported and collected only at baseline. The timing of blood collection during pregnancy was not standardized as maternal blood samples were collected during scheduled prenatal visits for convenience purposes. Determinations of plasma volume were not assessed in this research yet plasma volume expansion variability would be predicted to impact interpretation of all biomarkers measured during pregnancy. However at present there are no convenient ways to measure plasma volume. A few studies have tried to evaluate plasma volume across pregnancy using dyes or radiolabeled albumin, but either way is not approved for use in the U.S. (3-5). In this research, erythroferrone, a recently discovered hormone that mediates hepcidin

suppression, was not measured because there are currently no validated human assays for this hormone. Our study population had a high prevalence of ID, it is possible that some of the significant associations observed are only evident in women with depleted Fe stores and these findings may not be generalized.

5.2 FUTURE DIRECTIONS

This research provides normative observational data on Fe homeostasis in women carrying multiple fetuses and in their neonates. These findings have also generated new questions. Given the high prevalence of anemia and ID in women carrying multiple fetuses, it speaks to the need for more information on Fe homeostasis in this high-risk population. Mechanistic data on maternal Fe absorption and fetal Fe uptake are needed to identify Fe demands for women carrying multiple fetuses and their neonates, and to clarify the roles of fetally versus maternally-derived factors and their roles in placental Fe trafficking. Studies are also warranted to further determine the role of fetal hepcidin in regulation of placental Fe transfer and fetal Fe status. It is also important to determine if there might be key gestational windows to target in order to maximize fetal Fe acquisition across gestation.

This research, and a recent study in pregnant adolescents, identified an unexpected negative relationship between Hb and SF in the umbilical cord blood. The finding suggested that high cord SF concentrations at birth were associated with low cord Hb concentrations. More studies in different neonatal

populations are needed to understand the mechanisms behind this unexpected association.

Finally, these findings also speak to the need for more qualitative studies to understand dietary patterns and supplement compliance in women carrying multiple fetuses. Results from such work are necessary in order to design randomized control trials for Fe supplementation in women carrying multiples to determine whether an increased Fe intake would improve maternal and neonatal Fe status and birth outcomes in this high-risk groups.

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APPENDICES

PARTICIPANT CONSENT FORM AND STUDY DATA COLLECTION

APPENDIX 1. Participant Consent Form

Study Title: **Determinants of Neonatal Iron Homeostasis in Women Carrying Multiples**
Principal Investigators: **Kimberly O'Brien, PhD and Eva Pressman, MD**
Co-Investigators: **Ronnie Guillet, PhD, MD and Elizabeth Cooper, EdD, FACNM**

Introduction

This consent form describes a research study and what you might expect if you decide to participate. You should read this form carefully and ask the person who presents it any further questions you may have before making your decision whether or not to participate.
This study is being conducted by Eva Pressman, MD, Ronnie Guillet, PhD, MD, Beth Cooper, CNM, EdD and Kimberly O'Brien, PhD of the University of Rochester's Department of Obstetrics and Gynecology and Highland Hospital's Department of Obstetrics and Gynecology. Kimberly O'Brien is also on the faculty at Cornell University.
You are being asked to participate in this study because you are pregnant and between the ages of 19 and 50 years old and you are having either twins, triplets or quadruplets.

Purpose of the Study

The purpose of this research study is to learn more about iron status in women having twins, triplets and quadruplets, and to see how maternal iron status influences the amount of iron that is present in your babies at birth.

Background

Women who have too little iron in their bodies may have a greater risk of problems during their pregnancy. Iron is also very important for your baby while you are pregnant and the amount of iron infants have at birth has been linked to how children learn and develop. Pregnant women often do not get enough iron from their diet and they are at increased risk of anemia (too little hemoglobin in their blood) during pregnancy. This is most often due to iron deficiency. While iron deficiency is known to be a common problem during pregnancy, very little is known about iron status in women carrying twins or triplets. This study will measure how much iron you have in your blood and how much is stored in your body. By taking blood samples at different times during your pregnancy we will see how your iron status changes as your babies grow bigger and need more iron for their own bodies. We will also collect cord blood from your babies at birth to see how the amount of iron you have in your body is related to the amount of iron present in each of your babies at birth.

Description of Study Procedures:

If you agree to be in this study, we will ask you some questions prior to the study to see how healthy you are. We will also look in your medical chart to learn more about your health and to learn more about your pregnancy. You will be asked to give an extra tube of blood drawn each time you have blood drawn for clinically indicated tests over the course of your pregnancy. Results of testing conducted as part of this study and sub-studies will be included in your electronic medical record.
Pregnant women typically get blood samples taken for alpha-fetoprotein (at 16- 20 weeks), glucose tolerance testing (at 24- 28 weeks) and at delivery. At each of these visits you will be asked to allow us to take an extra tablespoon of blood from your arm (1 tablespoon is equal to 3 teaspoons or 15 mL of blood). We will use this blood sample to see how much iron is in your blood.

Second part of the study at delivery:

When you go into the hospital to have your babies, the nursing staff will take 1 tablespoon (15 ml) of blood from you as well as some blood from the cords of the placentas after your babies are born. The blood will give us information about your iron status and the iron status of your babies at birth. We will also take some samples from the placentas to measure proteins the placenta makes to help send iron to the babies while you are pregnant.

Number of Subjects

We plan to recruit a total of 100-120 pregnant women carrying multiples and obtain cord blood and placental tissue from their 200-360 babies at birth.

Risks of Participation:

A total of 3 tablespoons of blood will be taken during pregnancy. You may get a bruise and it may hurt a bit when the blood sample is taken. Some people feel lightheaded or faint when their blood is drawn. There is also a rare risk of infection. Samples of placenta and cord blood are also collected after the babies are born. Collection of these tissues poses no risk to you or to your babies.

A description of this clinical trial will be available on http://clinicaltrials.gov as required by U.S. Law. This website will not include information that can identify you. At most, the website will include a summary of the results. You can search this website at any time.

Benefits of Participation:

There is no direct benefit to you for participating in this study. You will receive information on your iron levels each time you have blood taken. These tests are not routinely taken at the time the other tests are done so this new information may be helpful to your doctor. If we see that you or your babies have too little iron (are anemic), we will tell you, your physician and your babies physician so that he/she can follow up with you about this if necessary.

Alternatives to Participation

You do not have to participate in this study if you do not want to. Your decision not to join this study will not affect the health care you receive at Strong Memorial Hospital or Highland Hospital.

Payments:

At the time of delivery when your babies are born we will give you a small gift for each of your babies and a $25 gift card to Target.

Sponsor Support:

The University of Rochester is receiving some support from the Gerber Foundation for conducting this research study.

Confidentiality of Records and HIPAA Authorization

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

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

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

We will use your health information to conduct the study and to determine how your health status and other medical care issues that are happening during your pregnancy might be influencing iron absorption and transfer to your developing baby. Health information is used to report results of research to sponsors and federal regulators. The health information collected may be audited to make sure we are following regulations, policies and study plans. URMC/Strong Health policies let you see and copy health information we have gathered for this research study after the study ends, but not until the study is completed. If you have never received a copy of the URMC/ Strong Health HIPAA Notice of Privacy Practices, please ask the investigator for one.

To meet regulations or for reasons related to this research, the study investigator may share a copy of this consent form and records that identify you with the following people: The University of Rochester; the Department of Health and Human Services; the United States Department of Agriculture; Cornell University; the University of Rochester; Highland Hospital; and your primary care provider.

If you decide to take part, your Authorization for this study will not expire unless you cancel (revoke) it. The information collected during your participation will be kept indefinitely. You can always cancel this Authorization by writing to the study investigator. If you cancel your Authorization, you will also be removed from the study. However, standard medical care and any other benefits to which you are otherwise entitled will not be affected. Canceling you Authorization only affects uses and sharing of information after the study investigator gets your written request. Information gathered before then may need to be used and given to others.

As stated in the section on Voluntary Participation below, you can also refuse to sign this consent/Authorization and not be part of the study. You can also tell us you want to leave the study at any time without canceling the Authorization. By signing this consent form, you give us permission to use and/or share your health information as stated above.

Contact Persons

For more information concerning this research, or if you feel you have suffered a research-related injury, please contact: Dr. Eva Pressman at Strong Memorial Hospital at (585) 275-7824 or Beth Cooper in the RAMP clinic at (585) 275-2962 or Kimberly O'Brien at (607) 255-3743.

Please contact the University of Rochester Research Subjects Review Board at 265 Crittenden Blvd., CU 420315, Rochester, NY 14642-8315, Telephone (585) 276-0005 or (877) 449-4441 for the following reasons:

- You wish to talk to someone other than the research staff about your rights as a research subject;
- To voice concerns about the research;
- To provide input concerning the research process;
- In the event the study staff could not be reached.

Voluntary Participation

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

Iron absorption and transport sub-study

We are conducting a sub- study to see how iron is absorbed and transported from the mother to babies in women carrying twins and triplets only. This sub-study is performed during your pregnancy and uses many measurements that are already performed in this study, though there are a few additional procedures. Would you like to hear more about this study? Your decision will not affect your participation in the Multiples study nor the health care you and your infants receive.

☐ Yes, I would like more information on the sub-study.

☐ NO, I am not interested in this sub-study.

☐ Patient is not eligible for sub-study

Infant auditory brainstem response sub-study

We are conducting a sub-study to learn more about how the iron supply to twins and triplets during pregnancy may affect the speed at which sound travels from the infant's ear to his/her brain. This sub-study will use data on iron that is already being obtained as part of the larger study. It will require and additional hearing test (called the auditory brainstem response) after your babies are born and before your babies leave the hospital. Your decision will not affect your participation in the Multiples study nor in the health care you and your infants receive. Would you like to hear more about this study?

☐ Yes, I would like more information on the sub-study.

☐ NO, I am not interested in this sub-study.

☐ Patient is not eligible for sub-study

Blood Samples

We would also like to collect cells from your blood and your babies' cord blood so that we can screen for DNA and genes involved in nutrient metabolism (how you process nutrients). The samples may be used to help identify genetic factors that influence nutrient metabolism in the body and to understand how these may be related to differences in iron status. The samples will not be sold or used directly for the production of commercial products. Reports about future research done with the sample will NOT be kept in your health records, but the sample reports may be kept with study records or in other secure areas. You can decide if you want your sample to be used for this type of research. Your decision can be changed at any time by notifying the study doctor in writing. Your decision about whether or not to provide this sample will not affect your participation in the iron measurement study or other studies.

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

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

Future Studies
We may want to contact you in the future regarding this study or to see if you and/or your children would be interested in participating in future studies. Your decision regarding future contact will not affect your participation in the Multiples study, nor will it affect your health care or your infants' health care in any way.

Please check one:

☐ No, I do not want to be contacted about this study or future studies.
☐ Yes, I agree to be contacted by the investigators in the future regarding this study or future studies:

Signed: _____

Date: _____

Signature/Dates

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

_____ Print Name

_____ Signature

_____ Date

Person Obtaining Consent

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

_____ Print Name and Title

_____ Signature

_____ Date

APPENDIX 2. Maternal Socio-Demographic and Health History Questionnaire

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Maternal / Fetal Bone Health In Pregnant Adolescents
Teen Bone Study - Health Survey Questionnaire

Demographics

MRN: ☐☐☐ - ☐☐ - ☐☐ DOB: ☐☐ / ☐☐ / 19☐☐ mm/dd/yyyy

Gestational Age: ☐☐ weeks* as of ☐☐ / ☐☐ / 20☐☐ mm/dd/yyyy*

When was the first day of your last period? ☐☐ / ☐☐ / 20☐☐ mm/dd/yyyy*

When is your baby due? ☐☐ / ☐☐ / 20☐☐ mm/dd/yyyy*

During what month / week of this pregnancy did you first seek prenatal care? ☐☐ Month OR ☐☐ Week

What was your average weight before pregnancy? ☐☐☐ lbs* OR ☐☐☐ kgs*

How tall are you without shoes on? ☐ ft ☐☐ inches OR ☐☐☐ centimeters

How old were you when you had your first period? ☐☐ years old

Is this your first pregnancy?

- O Yes
- O Don't know / Not sure
- O **NO**... How many past pregnancies, including this one?** ☐☐
  - How many children have you given birth to?** ☐☐
  - How many abortions have you had?** ☐☐
  - How many miscarriages have you had?** ☐☐

Do you intend to breastfeed your child? O Yes O No O Don't know yet/ Not sure

Were you using birth control? O No O Yes, using this type: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Have you had any of the following problems currently or in the past?

O Major Injuries O Auto Accidents O Broken bones O Bone disease O Joint Disease

Have you ever had a sexually transmitted disease (STD)?

- O No
- O Don't know / Not sure
- O **Yes, I have . . .** (bubble all that apply)
  - O Chlamydia O Bacterial vaginosis O Genital herpes O Syphilis
  - O Gonorrhea O Genital warts O HIV / AIDS O Trichomonas
  - O Other ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

*if unknown, code "?" (i.e. 11/??/2006) **if not applicable, leave empty.

Maternal / Fetal Bone Health In Pregnant Adolescents
Teen Bone Study - Health Survey Questionnaire

Have any of your family members had any of the following conditions? (grandparents, parents, brothers or children)

			Relationship
Diabetes	O Yes	O No	
Heart Disease	O Yes	O No	
High Blood Pressure	O Yes	O No	
Osteoporosis (brittle bones)	O Yes	O No	
Other bone / joint disease	O Yes	O No	

Medication		Frequency	Date (mm/dd/yy)
	Dose Unit		Start / / Stop / /
	Dose Unit		Start / / Stop / /
	Dose Unit		Start / / Stop / /
	Dose Unit		Start / / Stop / /

O Bubble here if there are more medications not listed.

Did you ever drink or currently drink alcohol?

O **Never**

O **Currently**
O **I Used To**
drink, . Cups
O Every day
O Occasionally
O 2 - 3 times a week
O Once a week
Since (mm/yyyy) /
Stop Date* (mm/yyyy) /

Did you ever smoke or currently smoke cigarettes?

O **Never**

O **Currently**
O **I Used To**
smoke . packs
Since (mm/yyyy) /
Stop Date* (mm/yyyy) /

Have you ever used and drugs such as marijuana, cocaine, stimulants, sedatives or other illicit drugs?

Drugs:	O marijuana	O cocaine	O stimulants	O sedatives	O narcotics	O diet pills	O other
Dosages:	O regularly	O regularly	O regularly	O regularly	O regularly	O regularly	O regularly
	O occasionally	O occasionally	O occasionally	O occasionally	O occasionally	O occasionally	O occasionally
	O unknown	O unknown	O unknown	O unknown	O unknown	O unknown	O unknown
Started* (yyyy)							
Stopped* (yyyy)							

*write "curr" or "__/curr" if drug is still in use. if unknown, code "?" (i.e. ??/200?) if not applicable, leave empty. (i.e no stop date)

Maternal / Fetal Bone Health In Pregnant Adolescents
Teen Bone Study - Health Survey Questionnaire

General Demographic Information

Are you currently covered by medical insurance or a health plan? O Yes O No

If **YES**, which plan?

Do you participate in the Women, Infants and Children (WIC) program? O Yes O No

Do you participate in any other public assistance programs? O Yes O No

If **YES**, which program?

Do you live alone? O Yes O No

If **NO**, who do you live with (bubble all that apply)?

O Mother O Father O Significant Other O Brother(s) O Sister(s) O Aunt / Uncle
O Cousin(s) O Friend O Grandparent(s) O Roomate O Other

What is your current marital status? O Single O Married O Divorced O Widowed
O Other:

What is your ethnicity? O Hispanic O Non-Hispanic

What is the ethnicity of the biological father of the baby? O Hispanic O Non-Hispanic O Unknown O Abstain

What is your race?

O American Indian or Alaska Native
O Native Hawaiian or Other Pacific Islander
O White / Caucasian
O Asian
O Black or African American
O Other

What is the race of the biological father of the baby?

O American Indian or Alaska Native
O Native Hawaiian or Other Pacific Islander
O White / Caucasian
O Asian
O Black or African American
O Abstain
O Unknown
O Other

What is your highest level of education completed? Years 0-6 = Primary School 7-12 = Secondary

Contact Information

Home Phone Number - -

Alternate Phone Number - -

Address

- -

Emergency Contact Phone Number - -

Relationship

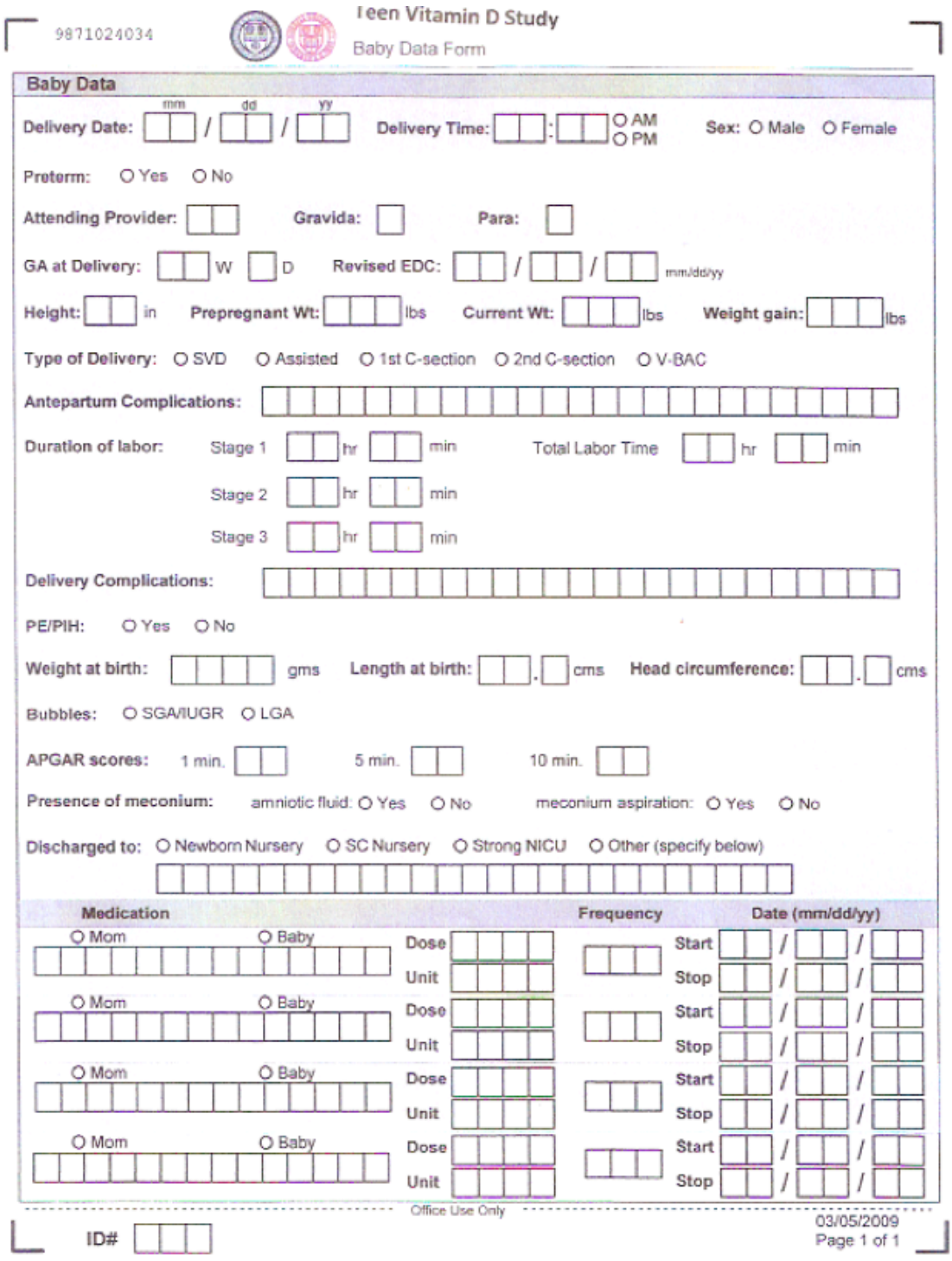
Name

Other

O Yes O No Relatives With Patient? Who?

ID# **Visit Date** mm/dd/yy / / **Visit** O 1 O 2 O 3

APPENDIX 3. Neonatal Birth Data



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Teen Vitamin D Study

Baby Data Form

Baby Data

Delivery Date: mm / dd / yy Delivery Time: : ○ AM ○ PM Sex: ○ Male ○ Female

Preterm: ○ Yes ○ No

Attending Provider: Gravida: Para:

GA at Delivery: W D Revised EDC: / / mm/dd/yy

Height: in Prepregnant Wt: lbs Current Wt: lbs Weight gain: lbs

Type of Delivery: ○ SVD ○ Assisted ○ 1st C-section ○ 2nd C-section ○ V-BAC

Antepartum Complications:

Duration of labor: Stage 1 hr min Total Labor Time hr min

Stage 2 hr min

Stage 3 hr min

Delivery Complications:

PE/PIH: ○ Yes ○ No

Weight at birth: gms Length at birth: . cms Head circumference: . cms

Bubbles: ○ SGA/IUGR ○ LGA

APGAR scores: 1 min. 5 min. 10 min.

Presence of meconium: amniotic fluid: ○ Yes ○ No meconium aspiration: ○ Yes ○ No

Discharged to: ○ Newborn Nursery ○ SC Nursery ○ Strong NICU ○ Other (specify below)

Medication		Frequency	Date (mm/dd/yy)
○ Mom ○ Baby	Dose / Unit		Start / Stop
○ Mom ○ Baby	Dose / Unit		Start / Stop
○ Mom ○ Baby	Dose / Unit		Start / Stop
○ Mom ○ Baby	Dose / Unit		Start / Stop

Office Use Only

ID#

03/05/2009

Page 1 of 1

APPENDIX 4. Maternal 24-Hour dietary Recall Questionnaire

Multiples Study
24 Hour Recall

_____ Subject Name

_____ DOB

_____ Subject Number

Date:_____

Time

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Was yesterday was a typical eating day for you: ☐ Yes ☐ No

If no, was this more or less food than you typically eat? ☐ More ☐ Less

Do you have any non-food cravings?

_____

APPENDIX 5. Eating Habit and Belief Interview Nutrition Questions

EATING HABITS AND BELIEFS INTERVIEW
NUTRITION QUESTIONS
Multiples Study

Subject name: _____ Date of interview:_____

M #:_____

1) Tell me about the foods and places you liked to eat most **before** you became pregnant.

2) Now I'd like to hear about any **changes** you've made to the foods you eat **now** that you are **pregnant**.

3) Can you explain to me what the words "**healthy eating**" mean to you?

4) What difference do you think it makes for the **baby** inside which foods you eat?

5) Who are the **people** you trust most to give you **advice** about what to eat during your pregnancy, and why? [Mother, partner, friends, prenatal care]

6) What other things influence the foods you eat?

7) What are the things in your life that make it more **difficult** to eat the foods you think are healthy?

8) How much **control** do you think you have over what you eat now and about changing what you eat? Tell me about that.

9) Do you eat meat?
   - If so what kinds of meat do you eat and how often do you eat these?