PHYSIOLOGICAL, ENVIRONMENTAL, AND GENETIC INFLUENCES ON IRON HOMEOSTASIS

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Iron deficiency is the most widespread micronutrient deficiency globally, while elevated iron stores are also associated with an increased risk of adverse health outcomes. Iron homeostasis must be strictly maintained to ensure that adequate iron is available for metabolic functions and to avoid the toxicity that results from iron overload. The overall goal of this research was to investigate selected physiological (pregnancy), environmental (parasitic infections and iron supplementation) and genetic (ethnic differences in iron status) influences on iron homeostasis.

Pregnancy places a significant strain on iron homeostasis. Surprisingly, there are very few longitudinal studies of hemoglobin concentrations across gestation and the norms that are used to define gestational anemia are based on limited evidence. To address a physiological determinant of iron homeostasis, this research provided a large normative analysis of determinants of maternal hemoglobin concentrations and identified factors associated with risk of maternal anemia in healthy U.S. pregnant women. Anemia was prevalent in otherwise healthy U.S. pregnant women even with the current universal iron supplementation recommendations. The existing CDC anemia cutoffs may overestimate the prevalence of anemia in late pregnancy based on our findings that were obtained in a significantly larger and more racially diverse population. Risk factors for anemia were identified as Black race, being underweight at entry into pregnancy, being multiparous or carrying a multiple birth pregnancy.

Multiple environmental factors can influence iron status and risk of iron deficiency and iron deficiency anemia. Parasitic infections are one example of a common environmental factor that can cause anemia. To obtain more detailed information on the impact of parasitic infections and iron supplementation, stable iron isotopes were used to investigate iron absorption and change in erythrocyte iron isotope enrichment in a group of toddlers residing in a malaria and helminth endemic environment. Toddlers who received iron supplementation exhibited greater loss of erythrocyte iron isotope enrichment over time, which may be indicative of an increase in the size of the erythrocyte pool and increased erythropoietic drive due to the provision of iron among anemic or helminth infected toddlers.

This study also summarized published literature on genetic determinants of iron status, characterized iron status in different ethnic populations, and statistically evaluated differences in iron status as a function of ethnicity using published data. A review of the literature identified multiple iron-related and non-iron related genes that have been associated with iron status. For the first time, these published data were used to statistically explore differences in iron status among various ethnic populations. East Asians had higher iron status compared to other racial groups yet this group also has the lowest prevalence of HFE mutations that are commonly associated with elevated iron stores. Future research using multidisciplinary approaches that consider physiological, environmental, and genetic factors is needed to better predict iron status at the individual and population level and to help develop the most effective interventions to maintain adequate iron status.

BIOGRAPHICAL SKETCH

Wanhui Kang was born in Taiyuan, China on November 9th,1993 to Fang Wang and Hongbing Kang. Wanhui graduated from Taiyuan No. Five Middle School in 2012 and was accepted to China Agriculture University in Beijing, where she majored in Food Quality and Safety. In her junior year, she was selected by China Agricultural University to study abroad at Cornell University, and she changed her major to Nutritional Sciences because of her passion for human nutrition. In June 2017, Wanhui graduated from Cornell University *summa cum laude* as Banner Bearer and from China Agricultural University and obtained dual bachelor's degrees. After graduating, Wanhui entered the doctoral program in nutrition in the Division of Nutritional Sciences at Cornell University, where she joined Dr. Kimberly O'Brien's research group. Her dissertation focuses on understanding the impact of pregnancy, environmental exposures, and ancestry on iron homeostasis.

DEDICATION

This dissertation is dedicated to my mother, 王芳 (Fang Wang) and my grandparents 王文华 (Wenhua Wang) and 连国英 (Guoying Lian). I love you forever.

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LIST OF ABBREVIATIONS

CRP	C-Reactive Protein
DMT1	Divalent Metal Transporter 1
EPO	Erythropoietin
ERFE	Erythroferrone
FPN	Ferroportin
Hb	Hemoglobin
HIF	Hypoxia-Inducible Factor
HJV	Hemojuvelin
ID	Iron Deficiency
IDA	Iron Deficiency Anemia
IL-6	Interleukin 6
IRE	Iron Responsive Element
IRP	Iron Regulatory Protein
LBW	Low Birth Weight
MT-2	Matriptase-2
NTBI	Non-Transferrin Bound Iron
RBC	Red Blood Cell
SF	Serum Ferritin
SGA	Small for Gestational Age
sTfR	Soluble Transferrin Receptor
TSAT	Transferrin Saturation
TBI	Total Body Iron
TfR	Transferrin Receptor
TIMS	Thermal Ionization Mass Spectrometry
TMPRSS6	Transmembrane Serine Protease 6
ZIP 14	Zrt/Irt-like protein-4

CHAPTER 1

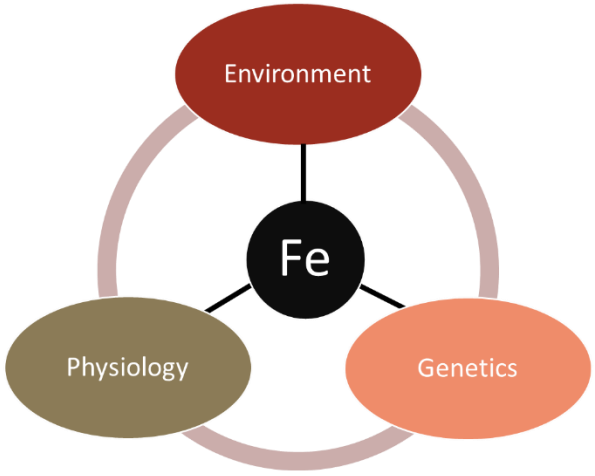
INTRODUCTION

Specific Aims

Iron is an essential trace element in almost all living organisms as it is required for a variety of metabolic and catabolic processes (1). Iron deficiency (ID) is the most widespread micronutrient deficiency, affecting an estimated 2.5 billion people worldwide (2,3). At the other extreme, iron excess is also detrimental to health as this leads to the generation of free radicals that can cause oxidative damage. Human epidemiological studies have documented that elevated iron stores are associated with an increased risk of developing type II diabetes (independent of inflammation) (4,5), cardiovascular disease (6,7), liver fibrosis, cancer (8–10), and neurodegenerative diseases (11,12). Iron balance must be strictly maintained to ensure that adequate iron is available for metabolic functions and to avoid the toxicity that results from iron overload.

Unlike other essential micronutrients, iron is unique because there is no regulatable excretory pathway to eliminate excess iron once it has been absorbed. Thus, iron homeostasis in humans depends almost exclusively on the tight regulation of dietary iron absorption by enterocytes. There are three major factors that influence the regulation of iron homeostasis, namely environment, genetics and physiological state (**Figure 1.1**).

Figure 1.1 Three major factors that influence iron homeostasis.



Physiological stages such as infancy, childhood, adolescence and pregnancy have a significant impact on iron homeostasis. Iron requirements change because of physical growth during childhood, increased physiological losses of iron in women due to menstrual blood loss, and due to the increased maternal and fetal iron demands of pregnancy. During pregnancy, physiologic iron demands increase substantially to support fetoplacental development and maternal adaptations to pregnancy.(13) Extremes of iron status during pregnancy (both maternal anemia and high hemoglobin (Hb) concentrations) have been reported to increase the risks of unfavorable birth outcomes such as small-for-gestational-age (SGA), preterm birth, and perinatal death (14). However limited normative data in healthy U.S. women with uncomplicated pregnancies are available to establish cut-offs for maternal Hb concentrations across pregnancy.

Many environmental factors influence iron status. Among these, parasitic infections caused by malaria or geohelminths can lead to inflammation-mediated increases in serum hepcidin. This chronic increase in hepcidin leads to a sequestration of iron in macrophages and decreased intestinal dietary iron absorption, thereby increasing the risk of anemia (15). Another

important environmental factor that impacts iron status is iron intake from diet and/or supplements. Adequate dietary or supplemental iron can help ameliorate ID and iron deficiency anemia (IDA). However, there are situations where administration of supplemental iron can be detrimental. One example is the use of supplemental iron for children residing in malaria-endemic areas (16). Studies conducted in malaria-endemic areas have found that untargeted oral iron supplementation increases the risk of contracting infectious diseases and led to increased mortality among preschool children (17,18). Evidence addressing mechanisms whereby helminth and malarial infections interact to impact iron absorption and response to supplemental iron treatment in affected children are not fully understood. To date little is known about iron absorption and utilization in children with mild chronic parasitic infections (19). In addition, the effects of oral iron supplementation on iron utilization and long-term iron metabolism in children residing in parasite-endemic areas is unknown.

A third factor that influences the regulation of iron homeostasis is genetic background. To date only about 30% of the interindividual variability in iron absorption can be captured using measures of systemic iron status or by iron regulatory hormones such as hepcidin (20–24). Variability in iron status and absorption in healthy populations may be attributed to genetic factors. While there are well known ethnic differences in risk of iron overload, no studies to date have summarized published data on genetic variants found to be associated with iron metabolism across different ethnic groups nor have published data evaluated iron status as a function of ethnicity.

To address existing gaps in knowledge on iron homeostasis, the goal of this dissertation is to improve our understanding of physiological, environmental and genetic influences on iron homeostasis. This dissertation will address the following specific aims and hypotheses:

Specific Aims and Hypothesis

Specific Aim 1: To characterize maternal Hb distributions and the prevalence of anemia in a large cohort of pregnant women and to identify factors associated with risk of anemia in this cohort.

Hypothesis: The 5th percentile of the Hb distribution used currently by the CDC will be closer to the 50th percentile of the Hb distribution in a modern pregnancy cohort. Mean Hb concentrations per trimester will be impacted by race/ancestry. The prevalence of anemia will be highest in the third trimester of pregnancy and highest in Black women, while the prevalence of high Hb concentrations will be highest in the first trimester and among Asian and White women.

Specific Aim 2: To utilize a stable iron isotope approach to identify the impact of parasitic infections and iron supplementation on iron incorporation into red blood cells (RBC) and to identify variables associated with the change in RBC iron isotope enrichment over a 3-month period among a group of toddlers residing in a malaria and helminth endemic environment.

Hypothesis: Iron status and iron supplementation will be negatively associated with iron incorporation into RBC's. Toddlers with parasitic infections will exhibit higher RBC Fe incorporation and a greater loss of RBC iron isotope enrichment post-dosing.

Specific Aim 3: To review genetic determinants of iron status and to characterize iron status in different ethnic populations and to undertake a statistical evaluation of possible differences in iron status as a function of ethnicity using published data.

Hypothesis: Both iron- and non-iron related genes or single-nucleotide polymorphisms (SNPs) will be associated with variations in iron traits among healthy populations. The frequencies of certain mutations or SNPs will differ as a function of ethnicity. East Asian and White populations will have the highest risk of elevated iron status while Black populations will have the highest risk of low iron status.

Background and Significance

I. Iron homeostasis

a. *General Iron Physiology*

Iron is an essential trace element involved in numerous metabolic processes, including oxygen transport and utilization, cellular proliferation, DNA synthesis, neurotransmitter synthesis, and energy production (1,25). Major organs or systems involved in iron metabolism include the small intestine, bone marrow, liver, and reticuloendothelial system. Four key cell types play key roles in regulation of iron homeostasis including the enterocytes, erythrocytic precursors, macrophages, and hepatocytes. Iron is absorbed into the blood stream across the enterocytes, specifically those of the proximal small intestine. Absorbed iron then binds to its specific systemic iron transport protein, transferrin. The majority of transferrin bound iron is delivered to erythrocyte precursors in the bone marrow to be incorporated into Hb in newly

synthesized RBCs (26). The distribution of total body iron is as follows: approximately two thirds of total body iron is contained within the circulating erythrocytes as heme. Heme consists of an iron ion complexed within a porphyrin ring. This complex is an indispensable component of Hb. Approximately 10-25% of body iron is stored as ferritin primarily in the liver and reticuloendothelial macrophages. This iron reserve can be mobilized when dietary iron absorption cannot meet iron demands. The remaining 5-15% of body iron is present within the heme component of myoglobin in muscle tissues and in a variety of enzymes involved in oxidative metabolism (26–29).

Unlike other essential micronutrients, iron is unique because there are no regulatable excretory pathways to eliminate excess iron once it has been absorbed. Thus, iron homeostasis in humans depends almost exclusively on the tight regulation of dietary iron absorption by enterocytes. There are two forms of iron ingested from dietary sources: heme iron and non-heme iron, and these two forms of iron are absorbed using different mechanisms. Absorption of non-heme iron (from both animal and plant sources) is regulated in response to iron status, whereas absorption of heme iron (from meat based dietary sources) is not tightly regulated in relation to iron status. Non-heme iron accounts for most of the dietary iron ingested primarily as ferric iron (Fe^{3+}) or as ferrous iron (Fe^{2+}) or in plant sources, animal tissues, or from iron fortificants that have been added to food during processing. Dietary ferric iron must be first reduced to ferrous iron (Fe^{2+}) by a ferrireductase, duodenal cytochrome B, before it can be transported into the enterocyte by the divalent metal transporter 1 (DMT1) protein. Once ferrous iron (Fe^{2+}) enters the enterocyte, it can either be stored as ferritin or exported across the basolateral membrane of

the enterocyte via iron export protein, ferroportin (FPN). As iron is exported across the basolateral membrane of the enterocyte, the ferrioxidase, hephaestin, oxidizes ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) in order for iron to bind to serum transferrin to be transported throughout the blood stream to other body tissues.

Transferrin-bound ferric iron is taken up into target cells that express transferrin receptor 1 (TfR1) by receptor mediated endocytosis. Cellular expression of TfR1 is highest in erythroid tissue to support the iron demands of erythropoiesis. Under normal circumstances, approximately 30% of the iron-binding sites in the plasma transferrin pool are occupied (30,31). This value is expressed as a percentage called transferrin saturation (TSAT), i.e. the ratio of serum iron/total iron binding capacity. When TSAT exceeds 45%, iron begins to circulate free or bound to low molecular weight molecules (citrate, albumin) generating a potentially toxic iron species known as non-transferrin bound iron (NTBI). The pancreatic cells and hepatocytes have a high capacity to internalize NTBI via ZIP14, while cardiomyocytes take up NTBI using L-type calcium channel and T-type calcium channel (32–34). Excess NTBI can increase the intracellular labile iron pool causing the generation of reactive oxygen species and free radicals that can cause oxidative damage particularly in organs that express ZIP 14 (liver and pancreas) or in the heart.

Only 1-2 mg of absorbed iron per day is needed to offset the typical amounts of daily iron lost from cell desquamation. The majority of iron needed to meet the high (20-25 mg/day) iron demands of erythropoiesis is obtained from the daily catabolism of senescent RBC's (30,35). The heme iron that is released in the macrophages of the liver, spleen, and bone marrow is crucial for maintaining physiological erythropoiesis. In general, disruption of all pathways

mentioned above, especially iron absorption, would result in abnormal iron metabolism and utilization, ultimately leading to adverse or even detrimental health consequences.

b. *Regulation of Iron Homeostasis*

Humans have evolved sophisticated mechanisms to regulate non-heme iron absorption. Iron absorption can be regulated at the cellular level by specific iron trafficking proteins and is regulated systematically by several hormones. The enterocyte can sense cellular iron needs through the "iron regulatory proteins (IRP) and iron response elements (IRE)" system and respond by altering expressions of iron transporters involved in cellular iron uptake and egress. In response to low iron concentrations in the labile iron pool, IRP can either bind to specific IRE located in the 3' or 5' untranslated regions of mRNAs that encode for key iron transport proteins, allowing them to block or stabilize their transcription. For example, when little iron is available in the labile iron pool level, the IRP1 and IRP2 in the enterocytes would bind to 5' region of the mRNA to block the translation of iron export and sequestering proteins such as FPN and ferritin; IRP's can also bind to 3' end of mRNA to stabilize the translation of genes that code for iron uptake proteins, such as DMT1 and TfR. In iron-replete cells, Fe/S clusters convert IRP1 into cytosolic aconitase, while IRP2 undergoes iron-dependent proteasomal degradation (36).

Iron absorption and iron homeostasis can also be regulated systemically via three iron regulatory hormones: erythropoietin (EPO), hepcidin, and erythroferrone (ERFE). The first of these three hormones to be identified was erythropoietin (EPO), which was found in the 1950's (37). Erythropoietin (EPO) is produced mainly by the kidneys in response to cellular hypoxia and when EPO binds to the EPO receptor in bone marrow this stimulates RBC production.

Decreased oxygen concentrations are detected by the kidneys which serves to stimulate EPO production. This process is mediated by hypoxia-inducible factors (HIF's), which are transcription factors that respond to reduced tissue oxygen levels (38).

Hepcidin is a small cysteine rich peptide hormone discovered in 2000 (1,39). Hepcidin is the key regulator of iron absorption and homeostasis. It is synthesized and secreted by the liver in response to inflammation, iron stores, oxygen tension, or erythropoietic demands. This hormone binds to FPN, causing it to be internalized, phosphorylated, ubiquitinated, and degraded within lysosomes. When present at higher concentrations, hepcidin can directly block FPN without endocytosis (40). This interaction limits iron egress from cells into the circulation by reducing absorption of dietary iron, recycling of iron from senescent erythrocytes, and release of iron from body iron reserves.

Iron regulated hepcidin expression is mediated primarily through the BMP-SMAD pathway. Accumulated iron stores in the liver upregulate BMP ligands, which along with the constitutively active BMP type II receptors phosphorylate and activate BMP type I receptors. This activation results in the phosphorylation of SMAD1/5/8, a mechanism possibly mediated by endofin (41). Phosphorylated SMAD1/5/8 associates with cytoplasmic SMAD4 (42) and this complex translocates to the nucleus where it binds to BMP responsive elements in the hepcidin promoter, resulting in the activation of hepcidin transcription (43). Although several BMP ligands are expressed in the liver, only two of them (BMP2 and BMP6) have been shown to play a role in hepcidin expression (42). Membrane-bound hemojuvelin (HJV) functions as an essential BMP co-receptor that binds selectively to BMP6 ligands, which may be facilitated by

neogenin (44), to enhance SMAD phosphorylation. Whereas the inactive form of HJV, soluble HJV, may down-regulate hepcidin in a competitive way interfering with BMP signaling (45). Another key player in the regulation of the BMP-SMAD signaling pathway is the major histocompatibility complex class I like protein HFE. Under high concentrations of saturated transferrin, diferric-transferrin binds to TFR1 and displaces HFE from TFR1. HFE is then able to form a complex with TFR2 and possibly HJV to promote BMP-SMAD signaling (44). HFE also interacts with and stabilizes ALK3 to prevent its degradation, subsequently promoting BMP-SMAD signaling and hepcidin transcription (46). Conversely, under conditions of iron deficiency, the transmembrane serine protease 6 (TMPRSS6 or matriptase-2, MT2) exhibits higher stability (47) and cleaves HJV into its inactive form, which results in the inactivation of the BMP-SMAD signaling pathway and downregulation of hepcidin transcription. In addition, the immunophilin FKBP12 inhibits the BMP-SMAD pathway through its interaction with ALK2 to dampen hepcidin expression (48).

Increased erythropoietic demands result in the downregulation of hepcidin expression via the function of EFRE (49,50). ERFE is a physiologic erythroid regulator mainly expressed in EPO-stimulated erythroblasts in the bone marrow (51). Decreased oxygen concentrations are detected by the kidneys via the HIF system, which stimulates the production and secretion of EPO from the kidneys. Elevated EPO then upregulates ERFE likely via the JAK2-STAT5 pathway (51) and ERFE suppresses hepcidin expression by sequestering BMP2/6 and inhibiting the hepatic BMP-SMAD signaling pathway (51–55). The specific molecular mechanism of action of ERFE on hepcidin is evolving and further studies are needed to solidify and expand

what is currently known. Moreover, under hypoxic conditions, decreased oxygen tension leads to decreased hepcidin expression by stimulating the transcription of matripase (MT-2) and furin, which cleaves HJV into its soluble form (56), in response to HIF (56).

Inflammation impacts hepcidin production as hepcidin is an acute-phase protein whose expression is up-regulated in response to infection and inflammation caused by diseases or parasites. The underlying mechanism of this response is mediated by the inflammatory cytokine interleukin-6 (IL-6). IL-6 binds to its receptor and stimulates hepcidin synthesis via JAK2-STAT3 pathway in collaboration with BMP-SMAD signaling (42). Additionally, animal data show that hepcidin expression induction by inflammation can be potentiated by activin B via the activation of the BMP-SMAD signaling pathway (57). A brief schematic of hepatic hepcidin regulation signaling pathways is presented in **Figure 3.1**.

c. ***Assessment of Iron Status***

Accurate assessment of iron status is important to not only predict iron demands but also to effectively intervene when needed to combat both iron deficiency and iron overload. The WHO recommends the use of multiple indicators to assess iron status and suggests 5 measures to characterize iron status: Hb, zinc protoporphyrin, mean cell volume, serum TfR, and serum ferritin (SF) (58). A brief description of iron status indicators and advantages/disadvantages are presented in **Table 1.1**. Measurements of SF and TfR are considered to provide the best approach to evaluate the iron status of populations when inflammation is absent (58). Serum ferritin is a measure of body iron stores and low concentrations (<12-15 ug/L) of SF indicate that iron stores are depleted. When inflammation is present, however, SF is not a useful indicator of

iron stores because concentrations of SF increase as a result of the acute phase response even if iron stores are low. Soluble TfR (sTfR) reflects the intensity of erythropoiesis and the cellular demands for iron. An increase in sTfR concentrations indicates increased cellular iron requirements or insufficient iron supply. Although sTfR appears to be less impacted by inflammation when compared SF, sTfR concentrations increase with general inflammation, increased erythropoiesis from malaria infection, or RBC disorders (59). In addition to use of individual measures of SF or sTfR, the ratio of sTfR to SF is often used to evaluate iron status. The logarithm of the ratio of sTfR to SF concentrations is linearly related to total body iron (TBI) when expressed as mg/kg body weight (**Table 1.1**). This calculated value provides a quantitative estimate of the size of the body iron stores; when iron is present values > 0 mg/kg are obtained and individuals that are iron deficient values < 0 mg/kg are obtained (60). Similarly, because both SF and sTfR are impacted by inflammation, TBI is also influenced by inflammation as this measure is obtained using both SF and sTfR. To control for limitations in serum ferritin as a biomarker when inflammation or infection is present, acute phase proteins including C-reactive protein (CRP) and α-1-antichymotrypsin can be measured to adjust for inflammatory conditions (58,61). Although not all anemia is caused by iron deficiency, Hb concentrations can provide useful information about the severity of iron deficiency. Hemoglobin concentrations are a key indicator of functional iron deficiency (ID). Hemoglobin can be used to diagnose anemia and as an initial screening indicator for ID , however, Hb concentrations are also impacted by smoking status, altitude, race, stage of gestation, thalassemia, vitamin A deficiency, and deficiencies of folic acid and vitamin B12 in certain settings (58).

Table 1.1: Biochemical iron status indicators

Biochemical Indicator	What this Biomarker Reflects	Advantages	Disadvantages
Hemoglobin (Hb) (g/dL)	Measure of anemia, reflect amount of functional iron in body	Inexpensive; simple to measure; important health consequences	Low sensitivity/specificity; late indicator
Hematocrit (Ht) (%)	Proportional volume of RBCs in whole blood	Simple to measure	Same as Hb
Transferrin receptor (TfR) (μg/L)	Indicator of tissue iron availability. Reflects balance between cellular iron requirements and iron supply	Unaffected by inflammation	Lacks standardization, affected by the rate of erythropoiesis
Serum ferritin (μg/L)	Indicator of storage iron	Reflects iron status; well standardized	Acute phase protein, affected by inflammation and sub-clinical infection
Total Body Iron (TBI) (mg/kg)	- [log (serum transferrin receptor/serum ferritin) – 2.8229]/0.1207	Measure of full range of iron status	Same as TfR and ferritin
Hepcidin	Regulator of iron homeostasis	Production diminished when iron reserves depleted	Affected by inflammation and sub-clinical infection
Serum iron (ug/dL)	Iron bound to transferrin and ferritin (minor) in blood	Measure of iron supply to the bone marrow and other tissues	Varies diurnally and after meals; sample easily contaminated with iron from outside sources
Total iron binding capacity (TIBC) (μg/dL)	Total capacity of circulating transferrin bound to iron	Increased in iron deficiency; low in inflammatory disorders	Large overlap between normal values and values in iron deficiency
Transferrin saturation (%)	Serum iron/TIBC	Proportion of transferrin bound to iron	Same as serum iron and TIBC
Zinc protoporphyrin (μmol/mol)	Indicates lack of iron to developing RBCs	Useful in young children; whole blood or dried spots can be assayed	Increased in iron deficiency, inflammatory disorders, exposure to lead

d. *Stable Isotope Methodology to Measure Iron Absorption*

Given the unique role of iron absorption in iron homeostasis, a reliable and accurate method to measure iron absorption and utilization is needed. Stable iron isotope techniques are an invaluable and safe approach that can be used to measure oral iron absorption from different foods and to investigate factors impacting iron bioavailability. An isotope is an atom whose

nucleus contains the same number of protons but a different number of neutrons. Isotopes of the same element differ from each other in their atomic mass due to a variable number of neutrons. Iron isotopes can either be radioisotopes, which emit ionizing radiation, or stable isotopes which are non-radioactive, occurring naturally in fixed amounts in nature. Stable iron isotopes can be used as tracers, that are deliberately administered to humans to study the metabolism of iron. There are four naturally occurring stable iron isotopes and three of these (^{54}Fe, ^{57}Fe and ^{58}Fe) are found at natural abundances under 6% (5.85%, 2.11% and 0.28% respectively). The erythrocyte iron incorporation method is the most commonly used method to measure iron absorption. The enrichment of orally administered stable iron isotopes in Hb is used as a proxy for iron absorption because the majority (80% or 90%) of absorbed iron is incorporated into RBCs in healthy adults (62) or infants and children (19,63,64) within 14 days. Thus, blood samples can be collected 14-days after administration of stable iron tracer and the enrichment of stable iron isotopes in whole blood can be measured to assess iron absorption. Thermal ionization mass spectrometry (TIMS) is often utilized to quantify the amount of iron isotope incorporated into RBCs after first digesting the blood, drying down the blood digests; isolating iron from the digests using anion exchange chromatography; loading the extracted iron onto rhenium filaments and measuring all four stable iron isotopes using TIMS. More details of the stable iron isotope method are presented in **Chapter 3**.

II. Physiological influences on iron homeostasis

Different life stages and corresponding physiological states have a significant impact on iron metabolism and homeostasis. The alteration of regular iron homeostasis occurs primarily in

individuals with increased iron requirements such as infants and children, adolescents during their growth spurt, women during their childbearing years, pregnant women, and in the elderly who may have pathophysiological changes in organ function and an increased inflammatory burden.

The iron stores at birth are very important in maintaining adequate iron status over the first 6 months of life (65). The size of the iron stores at birth may be affected by birth weight, gestational age, timing of umbilical cord clamping and maternal iron status. Iron is transferred from maternal blood to the fetus with most fetal iron acquisition occurring during the third trimester. Early cord clamping and maternal IDA have a negative effect on iron status of newborn (65). Studies in human infants (66,67) and experimental animals suggest that the regulatory capacity of iron homeostasis is limited in young infants, mainly caused by a lack of regulation of DMT1 and ferroportin (68,69), and hyporesponsive to hepcidin (70). Assuming an infant is born with sufficient iron reserves, at about 4 months of age these iron stores are typically exhausted and the requirement for dietary iron increases to support the infant's rapid growth. Rapid growth with high iron demands places infants and young children at particular risk for IDA, especially those aged 6–24 months (71). Iron deficiency and IDA can have a significant impact on infants' and children's development and health including altering immune status, increasing risk of morbidity, growth retardation, and adverse effects on cognition that may or may not be reversible with iron treatment (71). Iron is necessary for normal anatomic development of the fetal brain, myelination, and the development and function of the dopamine, serotonin, and norepinephrine systems, and iron also modifies the epigenetic landscape of the

brain (72). Adolescents are another at risk group for developing IDA due to their rapid growth and increased iron demands of puberty, especially among adolescent girls due to menstrual losses. Imbalances between nutritional iron intake and physiologic blood loss through menses or gestational iron losses are the primary reasons for ID and IDA in women in childbearing age (71,73). Menstrual blood losses may range from 25–50 mg of iron per cycle depending on the women's Hb concentration and the individual pattern of menstruation, and losses can be substantially greater in women with menorrhagia (73).

During pregnancy, iron requirements increase substantially to support fetoplacental development and maternal adaptations to pregnancy. In total, blood volume increases ~1.5 L to facilitate the blood flow in the uterus and placenta for nutrient and oxygen delivery to the fetus and to blunt the effects of blood loss at delivery (74). Insufficient increases in plasma volume have been associated with pathologies such as intrauterine growth restriction and preeclampsia (13,75). Iron requirements increase as pregnancy progresses. In the first trimester of pregnancy, the iron requirements (~0.8 mg/d) are lower than observed among non-pregnant women (1 - 1.5mg/day) (76) because of the cessation of menses. As pregnancy advances, maternal RBC mass increases and placental and fetal growth accelerates, physiologic iron requirements rise to 3.0 - 7.5 mg/d in the third trimester (13,73). To meet these increasing iron requirements, maternal hepcidin may be actively suppressed during pregnancy to increase both dietary iron absorption and mobilization of iron from stores (13). In healthy human pregnancies, maternal hepcidin concentrations decrease in the second and third trimesters, thereby facilitating an increased nonheme and heme iron absorption (77) as gestation progresses and to allow iron to be

mobilized from stores into the circulation (13). Other than hepcidin, EPO and sTfR concentrations increase as pregnancy progresses to support expanding maternal erythropoiesis (78–80). Serum ferritin concentrations gradually decrease reaching their lowest concentrations in the third trimester due to hemodilution and iron mobilization from stores to support the demands of the placenta and fetus (13). In addition, in an animal model of pregnancy, both DMT1 and the ferrireductase duodenal cytochrome B concentrations have been found to be increased (81).

Although physiological iron requirements do not differ between adult and elderly men and post-menopausal and elderly women (82), there is growing evidence that iron metabolism is affected by the aging process. Hemoglobin concentrations have been reported to decline with advancing age, and this decline appears to increase after the age of 80, particularly in men (83). In addition, the prevalence of anemia increases with age within the elderly population. Anemia in the elderly may be caused by a number of physiological factors including reduced efficiency of iron absorption and chronic disease (84). Chronic low-grade inflammation in the elderly can lead to less efficient iron absorption under the regulation by acute phase protein, hepcidin (84). Moreover, moderate or severe kidney failure is one of the causes of anemia in elderly, due to compromised EPO production (85). Anemia in the elderly has adverse health implications including a decline in physical performance, cognitive impairment, increased susceptibility to falling, frailty, and mortality (86). On the other hand, there are potential adverse effects of elevated iron stores in middle-aged and older people. Elevated iron stores have been found to be associated with increased risk of chronic diseases such as heart disease, cancer and type 2 diabetes mellitus (84).

III. Impact of environmental factors on iron homeostasis

There are many external environmental factors that impact iron status and iron absorption and can lead to disorders of iron balance. Lifestyle factors such as smoking, and alcohol consumption have been reported to be associated with iron homeostasis. Studies have exhibited that cigarette smoking induced significant dysregulation of iron homeostasis in the lung and lower sTfR values have been found in heavy smokers (1,87). Smoking can result in chronic hypoxic conditions and compromised lung capacity. The Hb distribution curve is shifted to the right in smokers and this has been found to be positively associated with the intensity of cigarette use (88). Other hematological indicators such as the hematocrit, MCV, MCH, MCHC, and RDW levels have also been found to be elevated in smokers to offset their compromised lung capacity (89).

Alcohol consumption is another lifestyle factor influencing iron homeostasis. High concentrations of SF have been positively correlated with the degree of alcohol consumption, and even mild to moderate alcohol consumption has been shown to increase the prevalence of iron overload (1). These effects may be mediated by changes in hepcidin production as animal studies have demonstrated that alcohol can down-regulate hepcidin expression in the liver (90).

Since there are no regulated biological excretion pathways to rid excess iron from the body, phlebotomy is used as an effective approach to treat iron overload. Blood donation can therefore have a negative impact on body iron stores. Results from the REDS-II Donor Iron Status Evaluation Study indicate that iron depletion is common in frequent blood donors, who were found to have significantly lower SF concentrations than non-blood donors or people who

had not donated blood for the past two years (91,92). Ferritin levels in blood donors were found to be inversely correlated with the cumulated number of blood donations, inter-donation intervals and donation intensity (91–94).

Although only 1-2 mg/day of absorbed dietary iron is needed to offset the typical amounts of iron lost per day, long term dietary iron deficit would lead to ID and eventually result in IDA. At a population level, dietary iron absorption is generally considered to be one of the most important determinants of iron status (95). Dietary iron absorption not only depends on physiological requirements and existing body iron stores, but it is also influenced by the quantity, form of iron and other dietary components. Non-heme iron absorption exhibits significant variability between otherwise healthy individuals with values typically ranging from 2% to more than 20% (1,27). Iron absorption can be enhanced by dietary components including ascorbic acid, organic acids (malic, lactic and tartaric acids) and animal tissues (meat, poultry or fish) whereas it can be inhibited by calcium, phytate, tannins, other polyphenols, and soy proteins (96,97). Food components that are inhibitors of non-heme iron absorption generally bind iron in the gastrointestinal tract and prevent its absorption, while enhancers of iron absorption are food components that weaken or prevent iron binding to inhibitory compounds or work by reducing ferric iron to ferrous iron (1).

Oral iron supplementation is a cost-effective strategy to treat ID and IDA in addition to improving dietary iron intake and bioavailability. Iron supplementation can be targeted to high-risk groups such as children and pregnant women who have higher physiological iron demands. Randomized control trials have reported that iron supplementation can improve Hb

concentrations in infants (98) and iron-deficient or anemic children (99) and can improve iron status (indicated by SF) in non-anemic iron-deficient adolescent girls (100). In addition, oral iron supplementation can reduce the risk of maternal anemia and iron deficiency in pregnancy (101). On the other hand, excessive iron supplementation of infants may lead to increased risk of infection, impaired growth and disturbed absorption or metabolism of other minerals (65,102,103) and may lead to dysbiosis in the gut microbiome (104). There has been controversy regarding the effects of exogenous iron administration in children in parasitic-endemic areas (16,17), particularly among children who are iron replete (105). Studies conducted in malaria-endemic areas have found that untargeted oral iron supplementation was associated with increased risk of contracting infectious diseases and increased mortality among preschool children (17,18). This effect, however, was not found in Nepal where there is no malaria, thus iron supplementation in malaria-endemic areas may be risky (106).

Malaria is currently one of the most geographically widespread and life-threatening diseases caused by *Plasmodium* parasites that transmit malaria to people through the bites of infected female Anopheles mosquitoes. Malaria is responsible for the deaths of an estimated 0.6 million people per year (17). In 2019, there were an estimated 229 million cases of malaria worldwide, and children under 5 years of age were the most vulnerable group (107). Another common infection is soil-transmitted helminth infections, which can be caused by the main species of the roundworm (*Ascaris lumbricoides*), the whipworm (*Trichuris trichiura*) or by hookworms (*Necator americanus* and *Ancylostoma duodenale*). More than 1.5 billion people are infected with soil-transmitted helminth infections worldwide, with the greatest numbers

occurring in sub-Saharan Africa, the Americas, and East Asia. Over 267 million preschool-age children and over 568 million school-age children live in areas where these parasites are intensively transmitted (108). Children living in helminth- and malaria-endemic areas frequently suffer from chronic parasitemia and low-grade inflammation. These parasitic infections coupled with low dietary iron intakes increase the risk of IDA in many deprived areas.

Mechanisms by which malaria and helminth infection impair iron homeostasis are multifactorial. Malaria may exacerbate ID by sequestrating iron into malarial pigment (hemazoin) (109) and by blocking iron recycling from senescent RBCs. It may also shorten the RBC lifespan due to erythrophagocytosis of both parasitized and non-parasitized RBC's (110,111). Helminth infections can lead to chronic gastrointestinal blood loss from ingestion of RBCs by parasites or tissue trauma caused by parasites attachment and feeding, which increase the risk of ID and anemia (112). In addition, inflammation caused by malarial and helminth infections may further compromise iron absorption due to increased serum hepcidin concentrations by chronic inflammation (110,111). Because of the controversial effects of oral supplementation on children residing in parasites-endemic areas, the WHO recommends screening for ID before iron supplementation is initiated in these locations (113). In addition, WHO recommends provision of iron supplements to children, but this should be implemented in conjunction with measures to prevent, diagnose and treat malaria for optimal results on children's health. Iron supplementation combined with anti-malarial and anti-helminth medication is commonly used as an effective strategy for managing anemia in malaria-endemic areas (114).

IV. Genetic influences on iron homeostasis

Tight control of iron homeostasis is needed to prevent ID or iron overload but to date, only about 30% of the interindividual variability in iron absorption can be captured by or iron regulatory hormones or iron status biomarkers (20–24). Iron status as determined using SF, sTfR and Hb concentrations varies significantly by racial/ethnic groups and different geographic areas (115–119). Ethnic differences in iron status indicators have been reported in large epidemiological studies or large cohort studies over the past few decades. For example, the largest epidemiological study to date that evaluated iron stores as a function of ethnicity was the HEIRS (Hemochromatosis and Iron Overload Screening) study. This study recruited 101,168 primary care adults aged 25 years or older from the United States and Canada, and evaluated SF and TSAT as a function of ethnicity (self-reported as Hispanic, European, African American, Asian, Pacific Islander, Native American) (120). These epidemiological studies have found that Asians have higher adjusted means of serum ferritin and transferrin saturation compared to any other population group studied, even after excluding polymorphisms previously associated with iron overload (115,118) and African Americans have lower mean Hb concentrations and higher SF concentrations compared to Caucasians (121,122). Race-ethnic differences in the risk of ID or iron overload have also been reported as highlighted by the CDC cut-offs used to denote anemia (123).

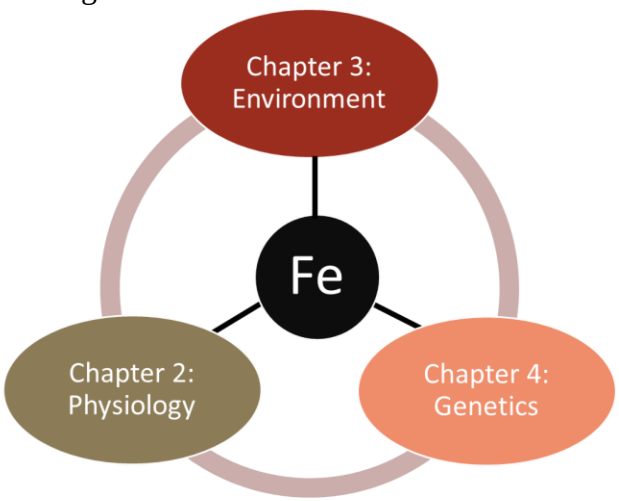
Findings from the O'Brien and Gu laboratories are in agreement with the epidemiologic data and have observed population-specific differences in iron absorption between East Asians and Europeans, and the differences noted persisted even after controlling for iron status (20).

These differences might indicate that the storage threshold at which East Asians down-regulate iron absorption is higher. Increased iron absorption and its impact on augmentation of body iron stores across age groups suggest that East Asian populations may be at greater risk of iron overload related conditions at maturity, which might explain East Asian populations having greater risk of diabetes at a lower BMI (124,125). Diet might be a plausible reason for the ethnic disparities in iron status because studies have found that there are associations between dietary constituents (such as red meat and vitamin C) and iron status (116,117). However, a randomized clinical trial conducted in elderly Europeans showed that changing from a Western to a Mediterranean-style diet for one year had no overall effect on iron status (116). On the other hand, genetic variations might underlie the observed ethnic differences in iron status. The HFE gene was first identified as the causative gene in hereditary hemochromatosis (HH) but the C282Y and H63D mutations of HFE that manifest at very high frequencies in Northern Europeans, are nearly absent in Asian and Pacific Island populations (119,126,127). In addition, a unique East Asian-prevalent HFE haplotype has been identified and may be a result of adaptive evolution (128). Clearly, there are unidentified genetic contributions that underlie the observed ethnic differences in iron status. Multiple genome-wide association studies (GWAS) and candidate gene association studies have been undertaken to investigate the genetic contributions to variations in iron status between and within populations. These studies have identified pathogenic mutations in some iron-related genes that associated with ID or iron overload and identified polymorphisms within iron- and even non-iron related genes that associated with variations in iron traits among healthy populations.

V. Significance and Summary

This dissertation project investigates three major factors that influence iron homeostasis: physiology, environment, and genetics. These three factors will be addressed in three specific aims of this dissertation that will be individually detailed and discussed in Chapters 2 through 4 (**Figure 1.2**).

Figure 1.2 Three major factors that influence iron homeostasis and the corresponding dissertation chapters addressing these factors.



For physiological factors, pregnancy will be focused on in this research. Maternal anemia has been reported to be associated with poor birth outcomes such as increased risks of low birth weight (LBW), SGA, preterm birth, stillbirth, perinatal and neonatal mortality (14,129) as well as adverse maternal outcomes including postpartum hemorrhage, preeclampsia, blood transfusion and maternal mortality (14,130). However, the policy of providing universal iron supplementation to all women in developed countries remains controversial. The US Preventive Services Task Force (USPSTF) determined that the current evidence was insufficient to recommend routine iron supplementation of pregnant women to prevent adverse maternal health

and birth outcomes (131). Limited normative data are available for maternal Hb in pregnancy and data to estimate the current prevalence of IDA among healthy US pregnant women. Therefore, Aim 1 (in **Chapter 2**) is designed to characterize the distribution of maternal Hb and the prevalence of anemia in a large cohort of pregnant women and to identify factors associated with risk of anemia in this cohort.

With respect to environmental factors and their impact on iron status, to date little is known about iron absorption and utilization in children with mild chronic parasitic infections (19). In addition, how oral iron supplementation impacts iron utilization and long-term iron metabolism in children residing in parasite-endemic areas is unknown. Therefore, Aim 2 (in **Chapter 3**) addresses the impact of malarial and helminth infections on iron incorporation into RBCs and identifies variables associated with the change in RBC iron enrichment over an 84-day period post dosing in a group of toddlers at high risk for malarial and helminth infections.

Lastly, understanding the impact of genetic polymorphisms and evolutionary origins on iron homeostasis may help guide dietary recommendations to minimize the risks of chronic diseases and guide future genome-informed nutritional practices. However, no published studies to date have summarized data on genetic variants that are associated with iron metabolism and highlighted how these genetic variants may differ across ethnic groups. Multiple published reports of iron status exist but few attempts have been made to compile these existing data to summarize current findings and knowledge on ethnic differences in iron status. Therefore, to fill this knowledge gap, Aim 3 (in **Chapter 4**) uses existing published data to statistically explore

differences in iron status among various ethnic/racial populations and to summarize and review published data on genetic determinants of iron status.

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CHAPTER 2

LONGITUDINAL CHANGES IN HEMOGLOBIN AND DETERMINANTS OF ANEMIA IN HEALTHY U.S. PREGNANT WOMEN*

*Kang W, Irvine C, Pressman EK, O'Brien KO. Longitudinal changes in hemoglobin and determinants of anemia in healthy U.S. pregnant women. *To be Submitted, The American Journal of Clinical Nutrition.*

Abstract

Background: Maternal anemia has been associated with poor birth outcomes. At present longitudinal data on hemoglobin concentrations across gestation are lacking and few data are available to estimate the current prevalence of anemia among U.S. pregnant women.

Objective: This study aimed to characterize normative maternal hemoglobin (Hb) distributions, the prevalence of anemia and determinants of anemia in a large cohort of healthy U.S. pregnant women receiving prenatal care at an urban medical center.

Methods: A 10-y retrospective medical chart review was undertaken of all healthy pregnancies that occurred between 2011 to 2020 at Strong Memorial Hospital and Highland Hospital in Rochester, NY. Data on Hb, serum ferritin and health and demographic information were abstracted from medical records. Mixed-effects logistic regression models were utilized to identify significant determinants of maternal anemia.

Results: A total of 54,453 pregnancies were identified, of these 41,226 were uncomplicated pregnancies that met all study inclusion criteria. Mean Hb concentration was lowest in the second trimester and the prevalence of anemia increased in each trimester of pregnancy ($P < 0.0001$). The overall prevalence of anemia was 16.7%, which significantly increased to 25.1% by late gestation. Risk factors for anemia included Black race or Hispanic ethnicity, being underweight at entry into pregnancy, being multiparous or carrying a multiple birth pregnancy. The current CDC anemia cutoffs which reflected the 5th percentile of the reference population represented the 5th, 16th and the 27th percentiles of our population in each trimester.

Conclusions: Anemia was evident in nearly 20% of otherwise healthy U.S. pregnant women and

the prevalence of anemia increased significantly as pregnancy progressed. Risk of maternal anemia was significantly higher in women that self-identified as Black race, or that were underweight, multiparous or carrying a multiple birth pregnancy. Current Hb cut-offs used to define anemia may be too low and may overestimate the prevalence of anemia.

Introduction

Maternal anemia is a significant public health problem that is estimated to have an global prevalence of 41.8% affecting nearly 56.4 million pregnancies (1). Anemia during pregnancy has been associated with poor birth outcomes including increased risks of low birth weight (LBW), small-for-gestational age (SGA), preterm birth, stillbirth, perinatal and neonatal mortality (2,3) as well as adverse maternal outcomes including postpartum hemorrhage, preeclampsia, blood transfusion and maternal mortality (2,4).

In the United States (U.S.), the 1999-2010 National Health and Nutrition Examination Survey (NHANES) reported an overall prevalence of gestational anemia of 2.6% (n = 1,283 pregnant women) (5), and based on the 2003-2012 NHANES data (n = 776 pregnant women) this prevalence increased to 8.8% (6). The trimester specific prevalence of anemia was reported using the NHANES 1999-2006 data which found that the risk of anemia increased nearly 5-fold as pregnancy progressed from 2.7% (n = 189), 2.2% (n = 416) and 10.8% (n = 384), in the first (T1), second (T2) and third (T3) trimester respectively (7). Of concern, the absolute number of pregnant women sampled in NHANES surveys has been relatively low, limiting the conclusions that can be generated from these cross-sectional data.

Iron supplementation is commonly used as an effective treatment to combat anemia among pregnant women and a review of this practice by the US Preventive Services Task Force (USPSTF) in 2015 concluded routine iron supplementation during pregnancy did improve intermediate maternal hematologic indexes, such as serum ferritin (SF) and hemoglobin (Hb). However this USPSTF review concluded that the existing evidence was insufficient to recommend routine iron

supplementation to pregnant women to prevent adverse maternal health and birth outcomes (9). The NIH Office of Dietary Supplements followed up on this report with a workshop highlighting evidence gaps and research needs on iron screening and supplementation in iron-replete pregnant women. One of the key knowledge gaps identified was limited cross-sectional data, and lack of longitudinal data on the prevalence of iron deficiency (ID) and iron deficiency anemia (IDA) among U.S. pregnant women (9). The existing U.S. Centers for Disease Control and Prevention (CDC) cut-offs for gestational anemia were derived from 5^{th} centile values obtained from longitudinal Hb data that was pooled from four small European studies undertaken more than 4 decades ago in healthy iron-supplemented pregnant women (n = 394) (10–13). The characteristics of current US pregnant women are dramatically different from the CDC reference populations in terms of racial composition, pre-pregnancy BMI (ppBMI) and supplemental iron intake (14).

To address existing gaps in this literature, we undertook a 10-year retrospective medical chart review of healthy pregnant women receiving prenatal care at a large academic medical center. Normative data on hemoglobin concentrations were abstracted from medical records to provide normative data on maternal Hb concentrations across gestation and to identify factors associated with risk of maternal anemia.

Methods

Study population and design

A retrospective medical chart review was undertaken using demographic and clinical data extracted from medical records of all pregnant women receiving prenatal care from 2011 to 2020 at Strong Memorial Hospital and Highland Hospital in Rochester, NY. Data were extracted from medical records by an honest broker and were provided in deidentified form to study investigators. This study was approved by the Institutional Review Boards of the University of Rochester and Cornell University.

Information on maternal demographic, anthropometric, and health-related characteristics were abstracted from the medical charts. Demographic information obtained included maternal age at delivery and self-reported race and ethnicity. Anthropometric data included maternal height measured during T1, self-reported pre-pregnancy body weight, and maternal weight across trimesters. BMI was calculated as weight [kg] / height^2 [m^2]. Women were classified into one of four BMI categories according to the CDC guidelines based on self-reported ppBMI as: underweight (< 18.5 kg/m^2); normal weight (18.5 to <25.0 kg/m^2); overweight (25.0 to <30.0 kg/m^2); obese, (30.0 to < 40 kg/m^2) or severely obese (≥ 40.0 kg/m^2) (17). Gestational weight gain (GWG) in kg was calculated as weight at the latest T3 measure obtained minus the pre-pregnancy weight. Health-related information included self-reported smoking status, whether women received any blood transfusions over gestation and if prenatal vitamins were prescribed during antenatal visits. Smoking status was reported as “never smoked”, “passive smoking”,

"quit smoking" or "currently smoking" based on information present in the medical records. In addition, data were also abstracted on gestational age at delivery, number of prenatal care visits, parity, multiple birth delivery, use of assisted reproduction technologies, delivery type (vaginal, C-section, vaginal birth after a cesarean section (VBAC), or other). Adequacy of prenatal care was determined using the Adequacy of Prenatal Care Utilization Index which was evaluated based on the timing of entry into prenatal care, total number of prenatal visits, and gestational age at delivery using Kotelchuck's method (18). Race was self-reported and classified into four categories as Asian (Asian, Asian Indian, Bangladeshi, Bhutanese, Burmese, Cambodian, Chinese, Filipino, Indonesian, Japanese, Korean, Laotian, Nepalese, Pakistani, Sri Lankan, Thai, Vietnamese), Black (Black or African American), White (White or Caucasian), or Other (American Indian or Alaskan Native, Native Hawaiian or Other Pacific Islander, Other, Other Pacific Islander, Samoan, Solomon Islander, Tahitian, Unknown or Patient Refused). Ethnicity was self-reported and classified as Hispanic, Non-Hispanic or Unknown.

All available Hb and SF data obtained across gestation were extracted from medical records. Hemoglobin analyses at the Strong Memorial Hospital and Highland Hospital were undertaken using the Cell-Dyn 4000 system (Abbott, Santa Clara, CA) in a CLIA certified laboratory. Serum ferritin concentrations were measured by Roche Cobas e601/e602 (Roche Diagnostics Rotkreuz, Switzerland). For women that had multiple Hb measurements obtained within the same trimester of pregnancy, the mean Hb concentration of the multiple values within each trimester was used for analysis purposes and the mean gestational age of the replicate measures was used as the time of measure. For women who had two Hb measurements (n = 154)

on the delivery date, the higher Hb value was used in data analyses. Anemia was defined using the CDC criteria as a Hb concentration < 11.0 g/dL in T1 or T3, or < 10.5 g/dL during T2. Data were also evaluated using race adjusted cutoffs to define anemia in Black women, whereby cut-offs were lowered by 0.8 g/dL as indicated by the CDC (19). Analyses were undertaken with and without this race adjustment factor. We also examined outcomes associated with elevated Hb concentration defined as either a Hb concentration greater than 13.0 g/dL (as Hb concentrations above this value have been associated with adverse health outcomes (2), or defined as a Hb concentration greater than 15.0 g/dL, as this was the 95th percentile of Hb concentrations based on data from the CDC reference population (19).

Statistical analyses

Descriptive statistics were used to summarize characteristics of pregnant women, as the mean ± standard deviation (SD), or median (interquartile range (IQR)) for continuous variables or counts and percentages for categorical variables. The distribution of Hb concentrations in each trimester was examined. Histograms were used to visualize the Hb distribution across each trimester and smoothed lines were fit to characterize distributions. Mean and standard deviation (SD) of Hb and the 3rd, 5th, 95th and 97th percentiles of Hb concentrations in each trimester were calculated. Possible differences in Hb concentrations between trimesters or sub-groups of the population were evaluated using mixed-effects linear regression models setting mother as the random effect to account for women that contributed more than one pregnancy over the 10-year study period. Least-square means were calculated in the post-hoc analyses when evaluating pairwise comparisons between trimesters. The overall p-values of the mixed-effects linear

regression models were calculated by F-tests. Possible differences in the prevalence of anemia and elevated Hb concentrations between trimesters were examined by mixed-effects logistic regression models. To identify risk factors associated with anemia and elevated Hb, mixed effects multiple logistic regression models were utilized controlling for trimester of pregnancy. The overall p-values of the mixed effects logistic regression models were obtained using Wald chi-square tests. Risk of anemia was evaluated using odds ratios (OR) with a 95% confidence interval. Statistical significance was defined as $P < 0.05$. All statistical analyses were conducted using R (version 4.0.3).

Results

Characteristics of the study population

Over the 10-year period from 2011-2020 a total of 54,453 pregnancies occurred. Of these, pregnancies that did not end in a live birth (n = 823), pregnancies that occurred to women diagnosed with diabetes (both gestational diabetes or type 1 or type 2 diabetes) (n = 3,829), autoimmune diseases (n = 121), HIV infection (n = 64), gastrointestinal disorders (n = 624), hemoglobinopathies (n = 385), hypertension including eclampsia and preeclampsia (n = 7,838), and thrombocytopenia (n = 130) were excluded resulting in a database that included 42,117 pregnancies that occurred to 31,158 women. The final data set was further cleaned to eliminate datapoints that were deemed to be biologically implausible and were attributed to data entry errors. These included Hb concentrations greater than 20.0 g/dL (n = 5) or less than 5.0 g/dL (n =

7), body weight < 35.0 kg (n = 16) or > 300.0 kg (n = 6), or height >2.0 m (n = 8) or < 1.2 m (n = 16). After further excluding healthy pregnancies without any Hb data available in the medical chart (n = 891), the final analytic sample included 41,226 births that occurred in 30,603 women (**Figure 2.1**).

Of all 54,453 pregnancies occurred between 2011-2020, 5% (n = 2,752) had no available Hb data, and of the 42,117 uncomplicated pregnancies, 2% (n = 888) had no Hb data in the medical record.

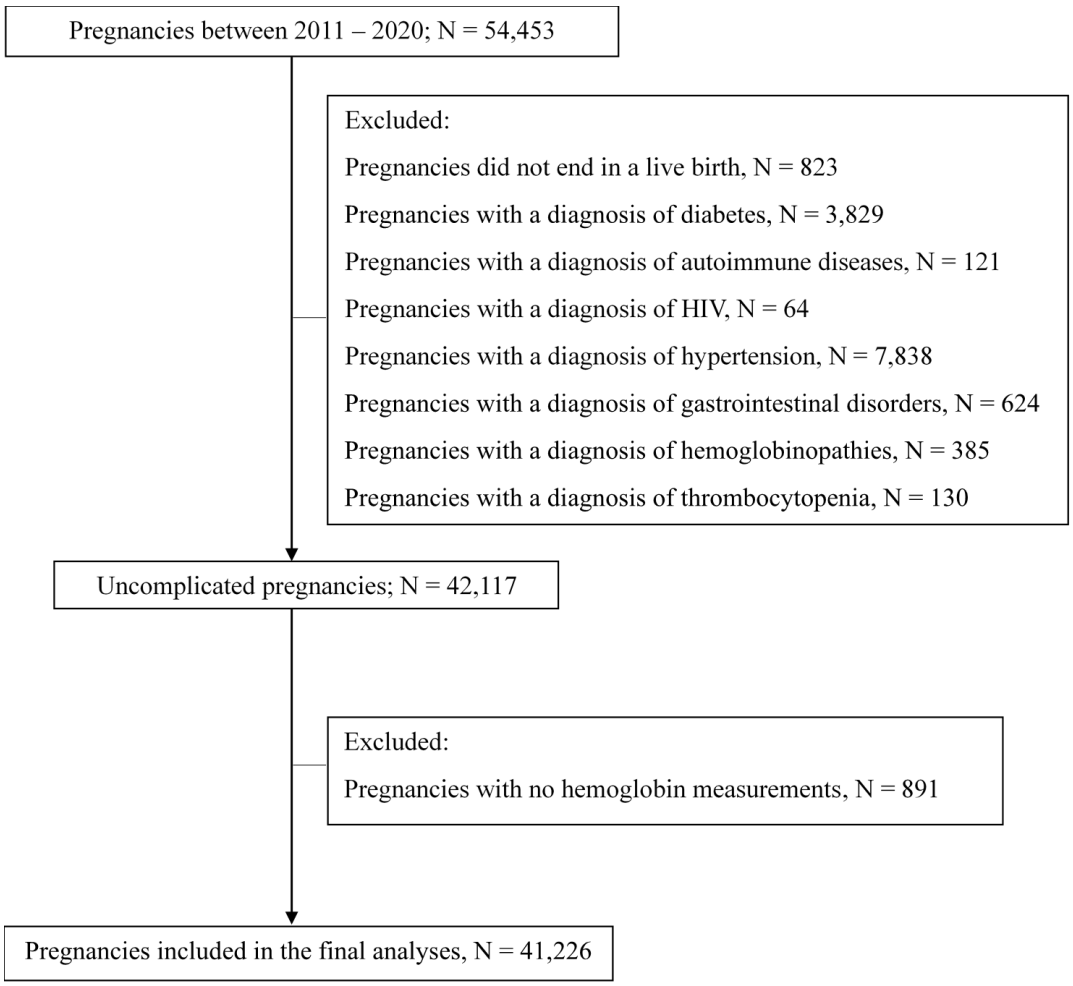

Figure 2.1 Flow chart of the study population identified from the retrospective medical chart review.

For pregnancies remaining in the final analytic sample, an average of 2.5 Hb measures were obtained across gestation. Of the Hb measures obtained, 28.6% were obtained in T1, 14.4% were obtained during T2 and 57.0% were obtained during T3. In our cohort, 78% of pregnancies had Hb measured in more than 2 trimesters: 4821 (12%) pregnancies had Hb measured in all three trimesters and 27,300 (66%) pregnancies had Hb measures in two trimesters. Of pregnancies that had longitudinal Hb measures (n = 32,121) in different trimesters of pregnancy, 0.5% (n = 168) had Hb measures in T1 and T2, 63.3% (n = 20,331) had Hb measures in T1 and T3, 21.2% (n = 6,801) had Hb measures in T2 and T3, and 15.0% (n = 4,821) had Hb measures in all three trimesters. Only 2.3% of the final pregnancy sample (n = 948) had SF data in their medical record. When obtained, the majority of SF analyses (41.2%) were obtained during T3, followed by T1 (30.2%) and T2 (28.5%). Among pregnancies with available SF data, 20% (n = 192) had more than one SF measurement obtained across pregnancy.

Characteristics of women in this study population are presented in **Table 2.1**. Only a small percentage of pregnant women receiving care were adolescents (≤ 19 y of age). The majority of women self-identified their race/ethnicity as White and non-Hispanic. The mean pre-pregnancy BMI fell slightly over the normal weight category, but the majority of women (74.5%) had missing pre-pregnancy BMI data in their medical chart (**Table 2.1**). Among all pregnancies with pre-pregnancy BMI data available, 44.4% of women were of normal BMI, 3.3% were underweight, 25.2% were overweight, 21.3% were obese and 5.9% were severely obese (≥ 40.0 kg/m^2) (data not shown). Almost half of the pregnancies with a known number of prenatal care visits (46.7%) were categorized as having Inadequate prenatal care based on the

Adequacy of Prenatal Care Utilization Index (**Table 2.1**). After preliminary analyses, the "currently smoking" (n = 3,097) and "passive smoking" (n = 180) groups had Hb concentrations that did not significantly differ and thus these were grouped together in the following analyses to increase the statistical power.

Table 2.1 Characteristics of pregnant women

Variable	Groups	Mean (SD) or Median (IQR)	N (%)
Age (y)		29.8 (5.5) *	41,226 (100)
	Adolescents < 20 y	18 (2)	1,405 (3.4)
	Adults ≥ 20 y	30.2 (5.2) *	39,821 (96.6)
Race		-	
	Asian	-	1,594 (3.9)
	Black	-	8,325 (20.2)
	White	-	27,417 (66.5)
	Other	-	3,890 (9.4)
Ethnicity		-	
	Hispanic	-	3,556 (8.63)
	Non-Hispanic	-	36,061 (87.5)
	Unknown	-	1,609 (3.90)
Pre-pregnancy BMI (kg/m^2)		25.4 (8.7)	10,507 (25.5)
	Underweight (< 18.5)	17.8 (1.0)	350 (0.9)
	Normal weight (18.5 - < 25)	22.0 (1.7) *	4,665 (11.3)
	Overweight (25.0 - < 30)	27.3 (1.4) *	2,643 (6.4)
	Obese (30.0 - < 40)	34.1 (2.8) *	2,233 (5.4)
	Severe Obesity (>= 40.0)	43.8 (5.4)	616 (1.8)
	Missing BMI data	-	30,719 (74.5)
Gestational weight gain (kg)		8.7 (5.7) *	-
	Underweight (< 18.5)	10.0 (5.3)	-
	Normal weight (18.5 - < 25)	9.8 (5.4)	-
	Overweight (25.0 - < 30)	8.9 (5.3) *	-
	Obese (30.0 - < 40)	6.3 (6.2) *	-
	Severe Obesity (>= 40.0)	3.6 (8.2)	-
Parity		1 (2)	41,174 (99.9)
	Nulliparous (Parity = 0)	0 (0)	16,330 (39.6)
	Primiparous (Parity = 1)	1 (0)	14,056 (34.1)
	Multiparous (Parity > 1)	2 (1)	10,788 (26.2)
	Unknown	-	52 (0.1)

Table 2.1 (Continued)

Use of assisted reproduction technology		
Yes	-	271 (0.7)
No	-	40,955 (99.3)
Blood transfusion during pregnancy	-	
Yes	-	588 (1.4)
No		40,638 (98.6)
Gestational weeks at delivery	39 (2)	41,226 (100)
Preterm (< 37 weeks)	34 (4)	3,533 (8.6)
Not preterm (≥ 37 weeks)	39 (1)	37,693 (91.4)
Multiple pregnancy	-	
No	-	40,278 (97.7)
Yes	-	920 (2.2)
Unknown	-	28 (0.1)
Delivery type	-	
Vaginal	-	29,593 (71.8)
C-section	-	10,801 (26.2)
VBAC	-	442 (1.1)
Other	-	38 (0.1)
Unknown	-	354 (0.8)
Adequacy of Prenatal Care Utilization Index		
Inadequate	-	6,640 (16.1)
Intermediate	-	1,000 (2.4)
Adequate	-	3,365 (8.2)
Adequate Plus	-	3,202 (7.8)
Unknown		27,019 (65.5)
Vitamins prescribed during pregnancy	-	
Yes	-	14,346 (34.8)
No	-	26,880 (65.2)
Smoking status	-	
Never	-	27,464 (66.6)
Passive or Yes	-	3,277 (8.0)
Quit	-	8,033 (19.5)
Not Asked or Unknown	-	2,452 (5.9)

* Data are presented as Median (IQR). VBAC, vaginal birth after a cesarean section.

Hemoglobin distributions among pregnant women

Table 2.2 summarizes Hb data, and the prevalence of anemia or elevated Hb in each trimester. Using all available Hb data, mean Hb averaged 12.0 g/dL; the prevalence of anemia

and Hb > 13 g/dL or Hb >15g/dL was 16.7%, 20.2% and 0.3% respectively. Because there were too few pregnant women with Hb concentrations greater than 15.0 g/dL, statistical analyses using this definition of elevated Hb were not used in further group analyses. The mean non-race adjusted Hb concentrations were significantly higher in T1 ($P < 0.0001$), compared to both T2 and T3 but Hb concentrations did not significantly differ between T2 and T3 ($P = 0.4$). The prevalence of anemia increased significantly across pregnancy, being highest in T3 ($P < 0.0001$) (**Table 2.2**). When anemia classifications were adjusted for race, the prevalence of anemia decreased by approximately 4 percentage points (i.e. from 16.7% to 12.8%). The 5^{th} percentiles of the observed Hb concentration in the group as a whole at T1, T2, and T3 was 11.0 g/dL, 9.8 g/dL and 9.6 g/dL respectively. The recommended CDC anemia cutoffs selected as the 5^{th} percentile of their reference population represented the 5^{th}, 16^{th} and 27^{th} percentile of our data distribution in each respective trimester (**Figure 2.2**).

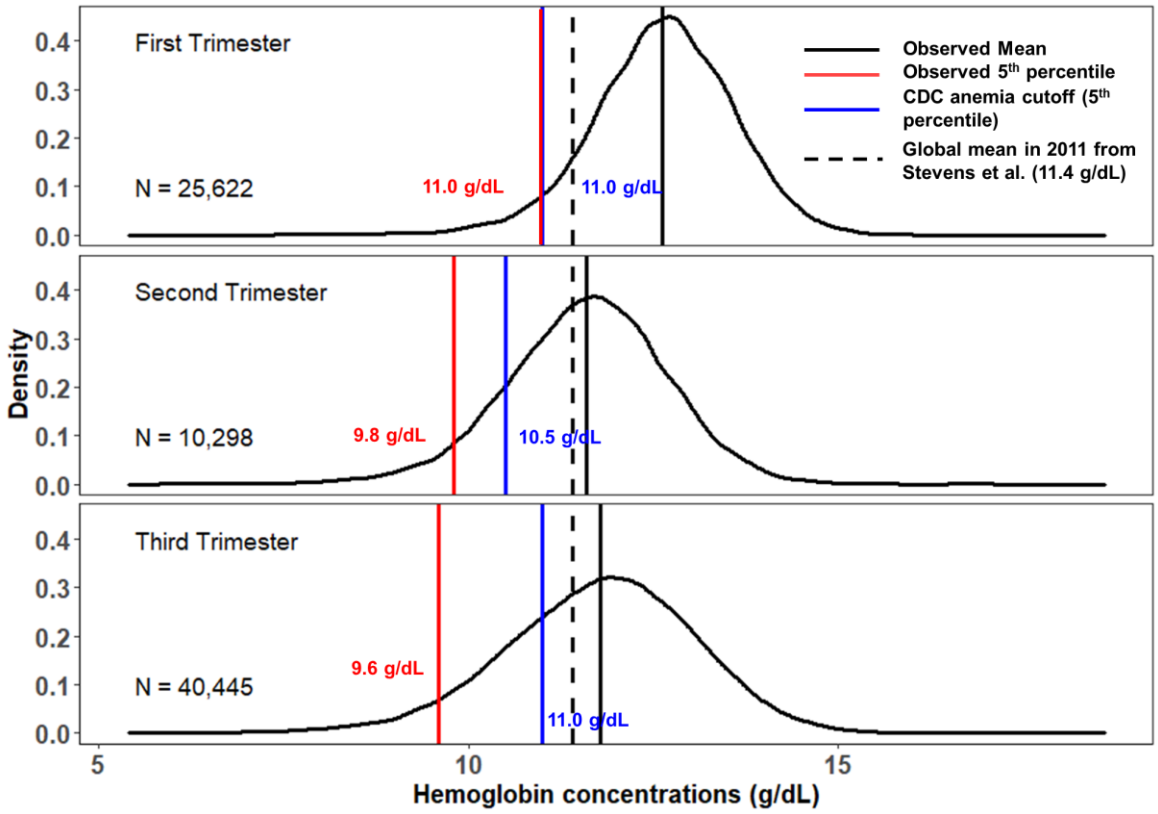

Figure 2.2 Hemoglobin distributions across gestation. Blue lines reflect the 5th percentile of the CDC reference data, red lines reflect the 5th percentile of our study population.

The 95th percentile (15.0 g/dL) of the CDC reference Hb distribution used to define an elevated Hb concentration was above the 99th percentile of the observed Hb concentration in our cohort. The highest observed Hb concentration was found at 4 weeks of gestation (13.1 g/dL) and the nadir of the Hb distribution was found at 30 weeks of gestation (11.0 g/dL). Values rose from 30 weeks of gestation until 40 weeks of gestation (**Figure 2.3**).

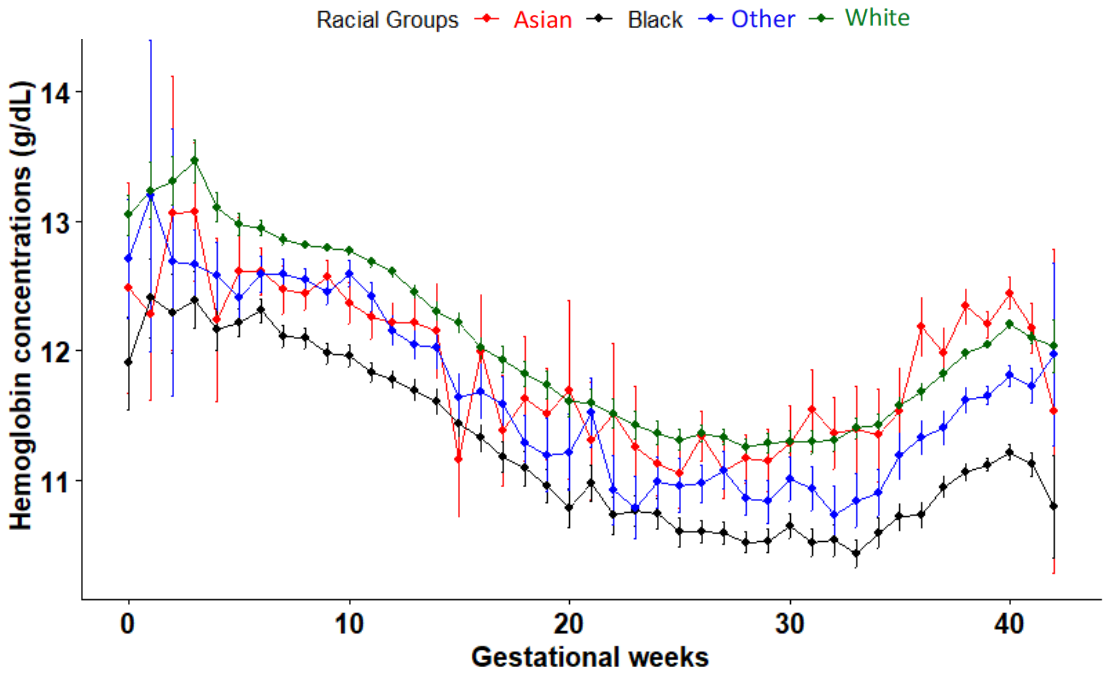

Figure 2.3 Hemoglobin concentrations across gestation. Dots reflect the mean hemoglobin concentration observed at each gestational week presented for each racial group. Error bars reflect the 95% confidence intervals of the mean.

Table 2.2 Hemoglobin concentrations and anemia/elevated hemoglobin prevalence across gestation

		Total	T1	T2	T3	P-value[1]
Hb concentrations (g/dL)						< 0.0001
	Mean	12.0	12.6	11.6	11.8	
	(SD)	(1.2)	(1.0)	(1.1)	(1.3)	
	Min	5.4	5.9	6.1	5.4	
	Max	18.6	18.6	16.9	16.6	
	3rd %ile	9.5	10.7	9.4	9.2	
	5th %ile	9.9	11.0	9.8	9.6	
	Median	12.1	12.7	11.6	11.8	
	95th %ile	13.9	14.1	13.3	13.8	
	97th %ile	14.1	14.3	13.6	14.1	
Anemia prevalence	%	16.7	4.5	14.0	25.1	< 0.0001
Anemia prevalence (race adjusted)	%	12.8	2.9	8.6	20.3	< 0.0001
Elevated Hb (Hb > 13 g/dL)	%	20.2	32.9	8.2	15.7	< 0.0001
Elevated Hb (Hb > 15 g/dL)	%	0.3	0.4	0.1	0.3	-

[1] Differences between hemoglobin concentrations among trimesters were analyzed by F-tests. Differences between anemia prevalence or elevated hemoglobin between trimesters were analyzed by Wald chi-squared tests. T1= first trimester; T2 = second trimester; T3 = third trimester.

Hemoglobin concentration comparisons between subgroups of study population

Hemoglobin concentration comparisons between sub-groups are presented in **Table 2.3**. Adult pregnant women had significantly higher Hb concentrations (+0.6 g/dL, $P < 0.0001$) than observed among pregnant adolescents. Mean Hb concentrations were significantly lower among Black women in each trimester on average by -0.8 (T1), -0.7 (T2), and -1.0 g/dL (T3) when compared to White women ($P < 0.0001$). White women had the highest Hb concentrations in T1 and T2 (12.8 ± 0.9, 11.9 ± 1.0 g/dL), while Asian women had the highest Hb concentrations in T3 (12.2 ± 1.2 g/dL, n = 1,577, $P < 0.0001$) (**Figure 2.3**). Black women had lowest Hb concentrations in all trimesters compared to other racial groups (12.0 ± 1.1, 11.2 ± 1.1, and 11.0 ± 1.3g/dL, $P < 0.0001$). In addition, non-Hispanic women (n = 3,556) had significantly higher Hb concentrations when compared to Hispanic women (n = 36,061) across gestation ($P < 0.0001$). Pregnant women with a normal or overweight ppBMI (n = 7,308) had significantly higher Hb concentrations ($P < 0.0001$) compared to means observed in underweight or obese women (n = 3,199). Pregnant women who were classified as receiving Inadequate prenatal care had significantly higher Hb concentrations ($P < 0.0001$) than other groups (**Table 2.3**). Interestingly, there were more White, Asian women ($P < 0.0001$), more non-Hispanic women ($P < 0.0001$), more nulliparous women ($P < 0.0001$) and more adolescents ($P = 0.0013$) were categorized as receiving Inadequate prenatal care. Pregnant women who stated that they never

smoked had significantly (P < 0.0001) higher Hb concentrations than women who smoked during pregnancy (**Table 2.3**).

Table 2.3 Mean hemoglobin concentrations as a function of key study variables

		Hb (g/dL)	Overall P-value[1]
Age (y)			< 0.0001
	Adolescent (< 20 y)	11.4 (1.2) [a]	
	Adult (≥ 20 y)	12.0 (1.2) [b]	
Race			< 0.0001
	Asian	12.2 (1.1) [a]	
	Black	11.3 (1.2) [b]	
	White	12.3 (1.1) [c]	
	Other	11.8 (1.2) [d]	
Ethnicity			< 0.0001
	Hispanic	11.8 (1.2) [a]	
	Non-Hispanic	12.0 (1.2) [b]	
Pre-pregnancy BMI (kg/m^2)			< 0.0001
	Underweight (< 18.5)	11.6(1.4) [ab]	
	Normal weight (18.5 - < 25)	11.9 (1.3) [c]	
	Overweight (25.0 - < 30)	11.9 (1.3) [c]	
	Obese (30.0 - < 40)	11.8 (1.2) [a]	
	Severe Obese (>= 40.0)	11.6 (1.4) [b]	
Parity			< 0.0001
	Nulliparous (Parity = 0)	12.2 (1.2) [a]	
	Primiparous (Parity = 1)	12.1 (1.2) [b]	
	Multiparous (Parity > 1)	11.7 (1.3) [c]	
Blood transfusion during pregnancy			< 0.0001
	Yes	11.0 (1.8) [a]	
	No	12.0 (1.2) [b]	
Gestational weeks at delivery			< 0.0001
	Preterm (<37 weeks)	11.7 (1.3) [a]	
	Not preterm (≥ 37weeks)	12.1 (1.2) [b]	
Multiple pregnancy			< 0.0001
	Singleton	12.0 (1.2) [a]	
	Multiples	11.6 (1.4) [b]	
Delivery type			< 0.0001
	Vaginal	12.1 (1.2) [a]	
	C-section	12.0 (1.3) [b]	
	VBAC	11.9 (1.3) [ab]	
	Other	11.9 (1.2) [ab]	

Table 2.3 (Continued)

Adequacy of Prenatal Care Utilization Index		< 0.0001
Inadequate	12.0 (1.3) [a]	
Intermediate	11.7 (1.3) [b]	
Adequate	11.9 (1.2) [b]	
Adequate Plus	11.8 (1.3) [b]	
Vitamins prescribed during pregnancy		< 0.0001
Yes	11.9 (1.3) [a]	
No	12.1 (1.3) [b]	
Smoking status		< 0.0001
Never	12.1 (1.2) [a]	
Quit	11.9 (1.2) [b]	
Yes or Passive	11.7 (1.3) [c]	

Data are presented as mean ± SD. Different superscripts indicate a significant difference in hemoglobin concentrations between sub-groups using mixed linear regression models. VBAC, vaginal birth after a cesarean section.

[1] Possible difference in hemoglobin concentrations between trimesters were evaluated using F-tests.

Anemia and high hemoglobin prevalence comparisons between subgroups of study population

In the group as a whole, nearly 1 in 5 pregnant women were anemic at one point over the course of gestation. Not surprisingly, factors that were associated with the prevalence of anemia (**Table 2.4**) were similar to the factors found to be associated with maternal Hb concentrations. The prevalence of anemia was highest among Black women in each trimester of pregnancy (13.6%, 24.2% and 47.6%), while White women had the lowest prevalence of anemia in the T1 (2.0%) and T2 (7.9%), Asian women had the lowest prevalence of anemia in T3 (15.3%). The prevalence of anemia increased by 8-fold from T1 (2.0%) to T3 (18.3%) in White women, by

2.5-fold (from 13.6% to 47.6%) in Black women, while only by 1.7-fold in Asian women (from 5.7% to 15.3%) (**Figure 2.4**).

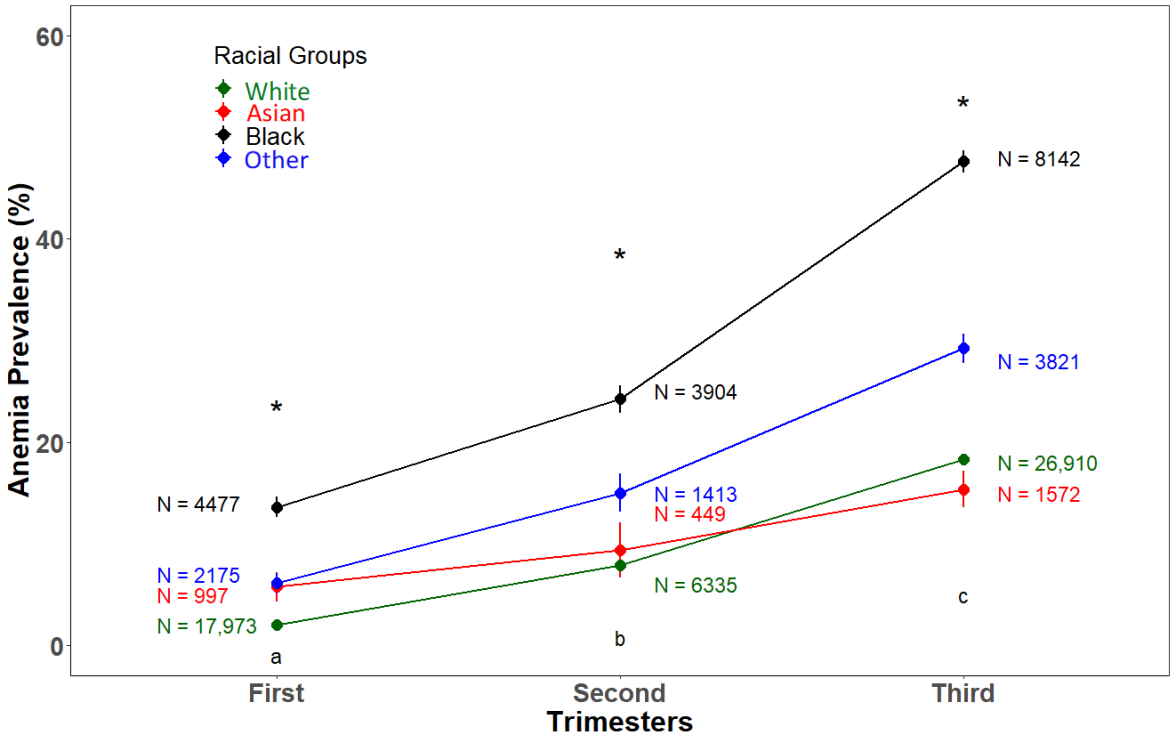

Figure 2.4 Self-reported racial groups and anemia prevalence. (*) indicates significant difference in anemia prevalence among racial groups in each trimester (P < 0.0001). (a, b, c) indicate significant difference in anemia prevalence among trimesters (P < 0.0001).

After race-adjusting the anemia cut-off for Black women, the prevalence of anemia remained significantly higher among Black women compared to Asian women and White women (P < 0.001) (**Table 2.4**). Women who were classified as underweight or severely obese based on ppBMI were 2 times more likely to be anemic compared to women with normal or overweight ppBMI (P < 0.001). Prevalence of obesity or severe obesity was 2 times higher among Black women compared to White women and prevalence of being underweight was 2.4 times higher among Asian women using ppBMI. Both ppBMI category and race were independently significantly associated with anemia. Multiparous and primiparous women were 4.6 and 2.3

times more likely to have anemia compared to nulliparous pregnant women (P < 0.0001). Women who were carrying a multiple pregnancy had almost 6-fold increased risk of gestational anemia compared to women carrying a singleton pregnancy (P < 0.0001). Unexpectedly, pregnant women who either smoked during pregnancy or were exposed to secondhand smoke were 2 times more likely to be anemic compared to women who had never smoked cigarettes (P < 0.0001). The comparisons of prevalence of elevated Hb between sub-groups are shown in the **Table 2.5**.

Table 2.4 Prevalence of anemia as a function of key study variables

		Anemia (%)	Pairwise P-value	Overall P-value	Anemia (race adjusted) (%)	Pairwise P-value	Overall P-value
Age (y)				0.80			0.17
	Adolescent (< 20 y)	30.2	-		19.5	-	
	Adult (≥ 20 y) (Ref)	16.2	0.80		12.6	0.09	
Race				<0.0001			<0.0001
	Asian	11.3	0.99		11.3	1.0	
	Black	32.9	<0.0001		14.5	0.0009	
	White (Ref)	11.3	-		11.3	-	
	Other	19.7	<0.0001		19.7	<0.0001	
Ethnicity				<0.0001			<0.0001
	Hispanic	21.6	<0.0001		20.3	-	
	Non-Hispanic (Ref)	16.3	-		12.1	<0.0001	
Pre-pregnancy BMI (kg/m^2)				< 0.0001			0.23
	Underweight (< 18.5)	26.9	0.0004		20.2	0.18	
	Normal weight (18.5 - < 25) (Ref)	18.7	-		14.1	-	
	Overweight (25.0 - < 30)	20.3	0.14		14.1	1.0	
	Obese (30.0 - < 40)	20.8	0.008		13.1	1.0	
	Severely Obese (>= 40.0)	26.4	<0.0001		15.3	0.92	
Parity				<0.0001			<0.0001
	Nulliparous (Parity = 0) (Ref)	13.0	-		9.8	-	
	Primiparous (Parity = 1	15.9	<0.0001		12.7	<0.0001	
	Multiparous (Parity > 1)	23.0	<0.0001		17.2	<0.0001	
Multiple pregnancy				< 0.0001			<0.0001
	Singleton (Ref)	16.4	-		12.5	-	
	Multiples	26.6	<0.0001		23.8	<0.0001	

Table 2.4 (Continued)

Adequacy of Prenatal Care Utilization Index			<0.0001		0.10
Inadequate	19.5	<0.0001		14.3	0.17
Intermediate	25.2	0.29		17.2	0.98
Adequate (Ref)	20.4	-		13.8	-
Adequate Plus	20.2	0.96		13.9	0.38
Smoking status			<0.0001		<0.0001
Never (Ref)	15.7	-		12.1	-
Quit	18.0	0.0006		13.7	<0.0001
Yes or Passive	23.4	<0.0001		16.9	0.02

Differences between anemia prevalence among sub-groups were analyzed by mixed logistic model from Wald chi-squared tests. (Ref) indicates the reference group for each group comparison.

Table 2.5 Prevalence of elevated hemoglobin concentrations (> 13.0 g/dL) as a function of key study variables

		Elevated Hb (Hb > 13) (%)	Pairwise P-value	Overall P-value
Age (y)				< 0.0001
	Adolescent (< 20 y)	8.9	-	
	Adult (≥ 20 y) (Ref)	20.6	< 0.0001	
Race				<0.0001
	Asian	21.1	0.0005	
	Black	6.8	<0.0001	
	White (Ref)	25.1	-	
	Other	15.3	<0.0001	
Ethnicity				<0.0001
	Hispanic	13.4	<0.0001	
	Non-Hispanic (Ref)	20.8	-	
Pre-pregnancy BMI (kg/m^2)				< 0.0001
	Underweight (< 18.5)	13.8	0.49	
	Normal weight (18.5 - <25) (Ref)	17.9	-	
	Overweight (25.0 - < 30)	16.9	0.80	
	Obese (30.0 - < 40)	13.9	<0.0001	
	Severe Obese (>= 40.0)	9.8	<0.0001	
Parity				<0.0001
	Nulliparous (Parity = 0) (Ref)	23.1	-	
	Primiparous (Parity = 1	21.0	<0.0001	
	Multiparous (Parity > 1)	14.8	<0.0001	
Multiple pregnancy				< 0.0001
	Singleton (ref)	20.3	-	
	Multiples	13.5	<0.0001	

Table 2.4 (Continued)

Adequacy of Prenatal Care Utilization Index				<0.0001
	Inadequate	18.3	0.0002	
	Intermediate	13.6	1.00	
	Adequate (ref)	16.2	-	
	Adequate Plus	16.4	0.72	
Smoking status				<0.0001
	Never (ref)	21.0	-	
	Quit	18.2	<0.0001	
	Yes or Passive	15.0	<0.0001	

Differences between elevated hemoglobin prevalence among sub-groups were analyzed using mixed logistic models from Wald chi-squared tests. (Ref) indicates the reference group for each group comparison.

Determinants of anemia during pregnancy

Determinants of anemia are presented in **Table 2.6**. Because 75% of pregnancies had missing ppBMI data, models were constructed including and excluding ppBMI as an independent variable. When maternal ppBMI was included in the model as an independent variable, variables found to be associated with a significantly higher odds ratio for anemia included Hb measurement during T2 or T3 of pregnancy; adolescent mother; Black or Other racial group, Hispanic ethnicity; being underweight; higher parity, carrying multiple fetuses, and smoking/passive smoking during pregnancy. In models that used race adjusted anemia cutoffs, maternal age, being of Black race or Hispanic ethnicity, and self-reported history of smoking during pregnancy were no longer statistically associated with anemia. When maternal ppBMI was excluded in the models, findings were similar to the results obtained using ppBMI, but pregnant adolescents and those who smoked during pregnancy had a significantly higher odds ratio for anemia using either the race-adjusted or non-race adjusted anemia cutoffs. The smoking

status association with Hb contradicts some findings on the impact of smoking on Hb concentrations reported in other studies carried out in non-pregnant adults. Cigarette smoking has been found to right shift the Hb distribution curve and Hb concentrations are often elevated in smokers compared with non-smokers as a response to the hypoxia caused by smoking (26). However, several previous studies in pregnant women have reported that smoking was associated with decreased Hb levels and increased risk of IDA (27–30). Although anemia risk factors including multiparity, underweight and being of Black race were associated with higher prevalence of smoking during pregnancy in our cohort, the odds ratio for anemia was still significantly higher in smokers after controlling for these variables. It is possible that other risk factors for anemia during pregnancy have a more pronounced effect on Hb concentrations, but our study was not designed to answer this question.

Table 2.6. Determinants of anemia

	ppBMI included as an independent variable (n = 10,279)				ppBMI not included as an independent variable (n = 37,416)			
	Anemia (R^2 = 0.67)		Adjusted anemia (R^2 = 0.98)		Anemia (R^2 = 0.77)		Adjusted Anemia (R^2 = 0.97)	
Variables	**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**
Trimesters								
First	Ref	-	Ref	-	Ref	-	Ref	-
Second	2.8 (2.3, 3.3)	< 0.0001	7.1 (5.2, 9.7)	< 0.0001	3.5 (3.1, 3.98)	< 0.0001	6.4 (5.4, 7.7)	< 0.0001
Third	20.6 (17.3 24.7)	< 0.0001	1,603.8 (1,128.6, 2,279.0)	< 0.0001	26.1 (22.5, 30.4)	< 0.0001	386.0 (324.4, 459.4)	< 0.0001
Maternal age at delivery (y)								
Adolescent (< 20 y)	1.9 (1.4,2.4)	< 0.0001	1.6 (0.8, 2.8)	0.13	2.0 (1.6, 2.4)	< 0.0001	1.7 (1.3, 2.4)	0.0008
Adult (≥ 20 y)	Ref	-	Ref	-	Ref	-	Ref	-
Race								
Asian	1.1 (0.8, 1.7)	0.52	1.1 (0.5, 2.5)	0.88	1.2 (0.9, 1.5)	0.15	1.2 (0.8, 1.8)	0.35
Black	7.4 (6.2 8.8)	< 0.0001	1.0 (0.7, 1.4)	0.94	13.2 (11.1, 15.7)	< 0.0001	1.2 (1.0, 1.5)	0.05
White	Ref		Ref		Ref		Ref	
Other	2.2 (1.7, 2.9)	< 0.0001	2.0 (1.16, 3.7)	0.02	2.4 (2.0, 2.9)	< 0.0001	2.0 (1.4, 2.8)	< 0.0001
Ethnicity								
Hispanic	1.3 (1.0, 1.7)	0.02	1.4 (0.8, 2.4)	0.21	1.7 (1.4, 2.1)	< 0.0001	1.7 (1.2, 2.3)	0.0009
Non-Hispanic	Ref		Ref		Ref		Ref	
Pre-pregnancy BMI (kg/m^2)								
Underweight (< 18.5)	1.7 (1.2, 2.3)	0.003	2.2 (1.1, 4.4)	0.03	-	-	-	-
Normal weight (18.5 - < 25)	Ref	-	Ref	-	-	-	-	-
Overweight (25.0 - < 30)	1.0 (0.8, 1.1)	0.83	0.9 (0.6, 1.2)	0.35	-	-	-	-
Obese (30.0 - < 40)	0.8 (0.7, 0.9)	0.006	0.7 (0.5, 1.1)	0.13	-	-	-	-
Severe Obese (>= 40.0)	1.2 (0.9, 1.5)	0.30	0.9 (0.5, 1.7)	0.79	-	-	-	-
Parity								
Nulliparous (Parity = 0)	Ref		Ref		Ref		Ref	
Primiparous (Parity = 1	1.9 (1.6, 2.2)	< 0.0001	2.9 (2.2, 4.0)	< 0.0001	1.8 (1.7, 2.0)	< 0.0001	2.6 (2.3, 3.0)	< 0.0001
Multiparous (Parity > 1)	2.8 (2.4, 3.3)	< 0.0001	4.7 (3.4, 6.6)	< 0.0001	3.0 (2.7, 3.4)	< 0.0001	4.6 (3.9, 5.4)	< 0.0001

Table 2.6 (Continued)

Multiple pregnancy								
Singleton	Ref		Ref		Ref		Ref	
Multiples	3.8 (2.6, 5.6)	< 0.0001	16.3 (6.3, 42.1)	< 0.0001	3.9 (3.1, 5.0)	< 0.0001	7.2 (5.0, 10.5)	< 0.0001
Smoking status								
Never	Ref		Ref		Ref		Ref	
Quit	1.0 (0.9, 1.2)	0.64	1.0 (0.7, 1.4)	0.90	1.2 (1.1, 1.3)	0.001	1.2 (1.0, 1.4)	0.11
Yes or Passive	1.3 (1.0, 1.6)	0.03	1.5 (1.0, 2.4)	0.07	1.6 (1.3, 1.8)	< 0.0001	1.5 (1.2, 2.0)	0.0006

ORs indicate adjusted odds ratio of anemia analyzed by the mixed effect multiple logistic regression models. (Ref) indicates the reference group for each group comparison. ppBMI, pre-pregnancy body mass index.

Discussion

This retrospective medical chart review of 41,226 pregnancies that occurred in a group of 30,603 healthy US pregnant women provided cross-sectional and longitudinal data on Hb concentrations across gestation. Anemia was evident in 16.7 % of these otherwise healthy pregnant women receiving standard prenatal care. The prevalence of anemia increased as pregnancy progressed, such that by late pregnancy fully 25% of women evaluated for anemia during the third trimester of pregnancy were anemic. Notably, the current CDC anemia cutoffs based on the 5^{th} percentile of the reference population in each trimester of pregnancy actually represented the 5^{th}, 16^{th} and the 27^{th} percentiles of our population in T1, T2 and T3 respectively. Risk factors for anemia included Black race or Hispanic ethnicity, being underweight at entry into pregnancy, being multiparous or carrying a multiple birth pregnancy. The prevalence of anemia across gestation was lowest among White and Asian women, and risk of elevated Hb concentrations was highest among White and Asian women.

The overall prevalence of anemia in this large cohort of women was 2 – 6.5 times higher than previously reported using national NHANES survey data (5–7). Evaluating data by trimester, the prevalence of anemia in each trimester remained more than 2 times higher than reported in the 1999-2006 NHANES survey (7). Although the 1999-2010 NHANES data only included singleton pregnancies, their pregnant population included a higher percentage of adolescents (6.3% vs. 3.4%) and a lower percentage of White women (53.5% vs. 66.5%) compared to our study population. In spite of having a greater percentage of women with these

two risk factors that we have identified as being associated with increased risk of anemia, the anemia prevalence they reported was markedly higher than that observed in our far larger cohort of women. The reasons for these discrepancies are unknown but may be impacted by the relatively small size of the NHANES study population (n = 1,283).

Notably, the current CDC anemia cutoffs that were identified as the 5th percentile of the reference population in each trimester of pregnancy reflected the 5th, 16th and the 27th percentiles of this much larger obstetric population in T1, T2 and T3 respectively. Recent Hb data obtained using pooled data from 257 population-representative data sources from 107 countries worldwide reported an average Hb of 11.4 g/dL in the group as a whole and from the figure presented in their paper it appears that the 5th percentile of their Hb distribution was also markedly lower than reported in the CDC data (**Figure 2.5**) (15).

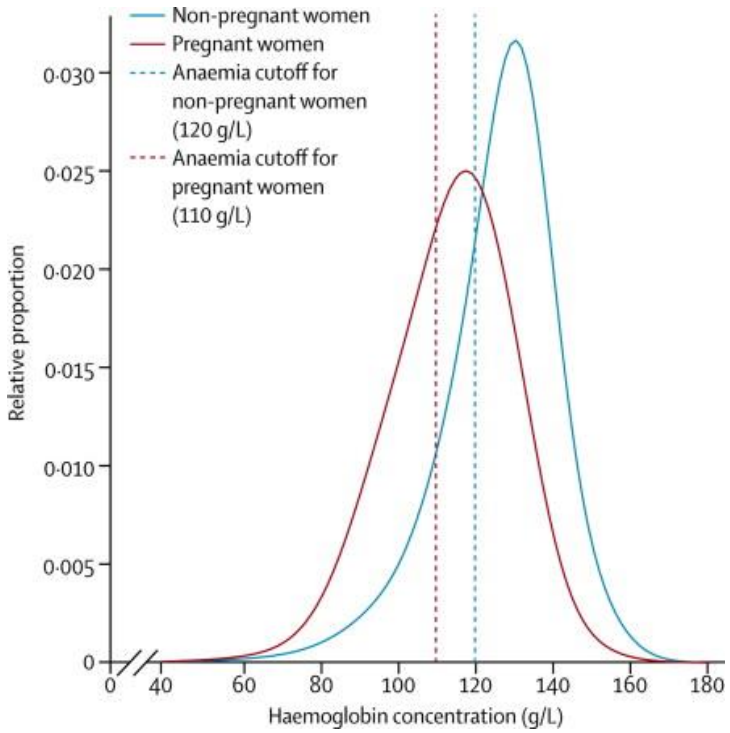

Figure 2.5 Global distributions of hemoglobin concentration for pregnant and non-pregnant women in 2011 presented by Steven et al.

The results of a recent international study presenting the Hb distributions in 3,502 healthy women pregnant carrying singletons also reported findings comparable to ours in that their reported 5th centile value at T2 and T3 (from 9.7 to 10.4 g/dL) were also lower than those currently utilized by the CDC (16). Hemoglobin concentrations higher than 9.5 g/dL-11.0 g/dL have found to be associated with higher risks of preeclampsia, prematurity, and fetal growth restriction (21), thus the current CDC reference anemia cutoffs may not be representative of current Hb distributions and if so this would overestimate the prevalence of anemia.

Interestingly, the 5th percentile of our cohort was the same as the CDC reference population in T1, but the Hb distribution shifted to the left in T2 and shifted to a larger extent in T3 in our population. One possible explanation might relate to the much larger dose of supplemental iron ingested by the CDC reference cohort. The European populations used to generate the CDC reference data ingested between 65 to 200 mg of supplemental iron per day which 2-7 times higher than the current US Recommended Dietary (27 mg/d) (14). In these European studies, the authors found that pregnant women receiving these high doses of oral iron supplementation during pregnancy maintained their iron stores and maintained normocytic erythropoiesis through T3, while compromised erythropoiesis was evident in non-iron supplemented women (10,11,13). We speculate that the current lower doses of iron supplementation may lead to the left shifted Hb distribution observed in our cohort. The CDC reference population was obtained in a cohort of women who were not representative of the current US pregnant population in regard to race/ethnic composition, ppBMI and supplemental iron intake (14). Larger more diverse cohorts of women that better resemble the racial and ethnic

composition and ppBMI distribution of the current US obstetric population, and that are ingesting supplemental iron intakes consistent with recent recommendations are needed to characterize anemia cutoffs during pregnancy and to help identify the current prevalence of anemia among US women.

Late gestation, Black race, multiple gestation pregnancy and multiparity were the strongest predictors of anemia during pregnancy. The anemia prevalence increased 3-fold across gestation, which is comparable to that reported in the NHANES 1999-2006 data (7). Racial disparities in Hb, with greater risk of anemia among women who self-identify as Black, have been identified in many previous epidemiological and population studies (5–7,22) and some CDC guidelines have suggested lowering the anemia cutoffs for Black women by 0.8 g/dL (19). The relative difference in Hb concentrations in our cohort of women are comparable to the CDC data as mean Hb concentrations were 0.8 g/dL lower in Black pregnant women compared to White women. Whether this difference in Hb concentration is a consequence of physiological changes or to differences in iron status requires further investigation and use of these race-specific cut-offs should be interpreted with caution in clinical settings to avoid the risk of under-diagnosing anemic women. We also noted that Asian pregnant women had the lowest prevalence of anemia across gestation, and the highest risk of elevated Hb concentrations in late gestation. Few data have evaluated Hb changes across gestation in Asian populations but the largest epidemiological study to date that evaluated iron stores as a function of ethnicity, the Hemochromatosis and Iron Overload Screening (HEIRS) Study, found Asians had the highest risk of elevated iron stores (23). Among women enrolled in the HEIRS study that were studied

while pregnant or breastfeeding, Asian ethnicity was also found to be associated with a decreased risk of ID (24). In agreement with these epidemiologic data, iron absorption has been reported to be significantly higher among non-pregnant East Asian women when compared to White women, even after controlling for iron stores as evaluated using SF (25). More data investigating the longitudinal changes in Hb across pregnancy and the impact of ethnicity on iron homeostasis are needed.

This study has some limitations. No data on birth outcomes were available and we are unable to address the impact of gestational anemia or elevated Hb concentrations during pregnancy on adverse maternal or neonatal birth outcomes. Data on maternal educational level and socioeconomic status were not available to evaluate the potential impact of these factors on risk of anemia or to compare the characteristics of this cohort to other published data on determinants of anemia. These pregnant women resided in Rochester, NY and the surrounding community (elevation of 214 meters above sea level) and these data would likely not be relevant for those living at high altitudes.

Our study provides the largest normative longitudinal datasets on Hb across pregnancy in otherwise healthy US pregnant women receiving routine prenatal care. These results add to the current knowledge on normative hemoglobin concentrations in US pregnant women and help identify determinants of anemia across pregnancy. We can conclude from this medical chart review that anemia was prevalent in otherwise healthy US pregnant women receiving standard prenatal care even with the current universal iron supplementation recommendations. The prevalence of anemia increased as pregnancy progressed. Risk factors for anemia included Black

race, being underweight at entry into pregnancy, being multiparous or carrying a multiple birth pregnancy. The hemoglobin distribution in the population as a whole was left-shifted in T2 and T3 compared to the CDC reference population but was similar to other recent global studies of hemoglobin in large pregnancy cohorts (15,16). Additional studies are needed to evaluate the impact of maternal anemia on iron status in the newborn at birth and its association with maternal birth outcomes to evaluate the current hemoglobin cut-offs used to define anemia. Targeted identification of women at greater risk may help inform interventions to improve maternal iron status and the in-utero environment in support of healthy birth outcomes.

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CHAPTER 3

IRON SUPPLEMENTATION IN ANEMIC ZANZIBARI TODDLERS IS ASSOCIATED WITH GREATER LOSS IN ERYTHROCYTE IRON ISOTOPE ENRICHMENT *

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Abstract

Background: Heavy parasitic loads increase the risk of iron deficiency anemia, which remains prevalent globally. Where parasites are common, understanding the influence of parasitic infections on Fe incorporation and erythropoiesis in toddlers is especially important.

Objective: The aim of this study was to identify the impact of malarial and helminth infections on red blood cell (RBC) Fe incorporation and subsequent change in RBC Fe isotope enrichment for 84 days post-dosing in toddlers at high risk for parasitic infections.

Methods: Fe incorporation was measured in a group of Zanzibari toddlers (n = 71, 16-25 months) using a stable Fe isotopic method. At study entry, oral stable Fe isotope was administered. Blood was collected 14 (D14) and 84 (D84) days post-dosing for assessment of Fe status indicators and RBC isotopic enrichment. Blood and stool samples were collected and screened for malaria and helminth parasites. Factors associated with change in RBC Fe isotope enrichment were identified using regression models.

Results: Toddlers who had larger weight-for-age z-scores, lower total body Fe and those with helminth infections (n = 26) exhibited higher RBC Fe incorporation. RBC Fe isotope enrichment decreased from D14 to D84 by -2.75 percentage points ($P < 0.0001$, n = 66). Greater loss in RBC Fe isotope enrichment from D14 to D84 was observed in those who received Fe supplementation, those with either helminths or both malarial and helminth infections, and in those with greater RBC Fe incorporation on D14.

Conclusion: Toddlers who received iron supplementation exhibited significantly greater loss of RBC iron isotope enrichment over time. We speculate this greater loss of RBC iron enrichment

is indicative of increased erythropoiesis due to the provision of iron among anemic or helminth infected toddlers.

Introduction

Iron deficiency and iron deficiency anemia (IDA) are particularly detrimental during the perinatal period and low iron stores at this time have been associated with long-term cognitive and developmental deficits (1). In populations with IDA, iron availability is not sufficient to meet erythropoietic demands. Toddlers living in helminth- and malaria-endemic areas frequently suffer from chronic parasitemia and low-grade inflammation. These parasitic infections coupled with low dietary iron intakes increase the risk of IDA in many developing countries. Mechanisms by which malaria and helminth infection impair iron homeostasis are multifactorial. Malaria may exacerbate iron deficiency by sequestrating iron into malarial pigment (hemozoin) (2) and by blocking iron recycling from senescent red blood cells (RBC). It may also shorten the RBC lifespan due to erythrophagocytosis of both parasitized and non-parasitized RBC's (3,4). Helminth infections can lead to chronic gastrointestinal blood loss increasing the risk of iron deficiency and anemia (5). In addition, inflammation caused by malarial and helminth infections may further compromise iron absorption due to increased serum hepcidin concentrations (3,4).

Iron supplementation can be used to treat iron deficiency and IDA, but concerns exist when providing supplemental iron to children residing in malaria-endemic areas (6,7), particularly if they are iron replete (8). In malaria-endemic areas, non-targeted iron supplementation has been associated with increased mortality of preschool children (7,9). For this reason, the WHO now recommends screening for iron deficiency before iron supplementation is initiated in malaria-endemic locations (10). In addition, iron supplementation combined with anti-malarial medication is commonly used as an effective strategy for managing

post-malarial anemia (11). However, the mechanisms of how helminth and malarial infections interact to impact iron absorption and response to treatment are not fully understood (12). A better understanding of the differences and cumulative impact of these infections on iron metabolism in children is needed.

This study was undertaken on Pemba Island, Zanzibar in 2004 when the overall prevalence of Plasmodium falciparum malaria was estimated to be 80% with no distinct evidence of seasonality (13,14). Moreover, among those aged 10-11 months, helminth and hookworm infections on Pemba island were endemic (13). The primary outcomes of this study were RBC iron isotope incorporation at D14 and changes in RBC iron enrichment over an 84-day period post dosing. The current study was undertaken to a) identify the impact of malarial and helminth infections on iron incorporation into RBC's in relation to iron status indicators, and b) to identify variables associated with the change in RBC iron isotope enrichment over an 84-day period post-dosing among a group of toddlers at high risk for malarial and helminth infections.

Subjects and Methods

Subject recruitment and sample collection

Participants were recruited to represent a random sample of healthy toddlers between 16 and 25 months of age with no other known health conditions. Toddlers that had fevers, symptomatic malaria, sickle cell trait or severe anemia (hemoglobin (Hb) < 7 g/dL) were not recruited into the study. A total of 82 toddlers were recruited from local villages on Pemba Island in November 2004. The stable isotope dosing study was initiated in December 2004 and ended in

March 2005. The study protocol was approved by the Committee of Human Research at the Johns Hopkins Bloomberg School of Public Health and the Ministry of Health, Zanzibar. Informed verbal consent was obtained from families willing to participate in the study. On the first day of the study parents were asked about their children's diet and recent illnesses. Each toddler received a physical examination and anthropometric z-scores were calculated using the WHO database (15). A finger prick was obtained to assess Day 0 (D0) Hb, and malaria parasitic count.

After D0 measurements were obtained, toddlers ingested a meal of porridge and remained fasted for 1.5 hours before ingesting a stable iron isotope as ferrous sulfate. Each participant received either 58Fe (0.9 mg of 58Fe, along with 6.0 mg of native ferrous sulfate to obtain a total iron dose of 7.0 mg, n = 37) or 57Fe (7.0 mg of 57Fe, n = 34) based on isotope availability. Tracer doses were administered with flavored raspberry syrup containing 0.391 % ascorbic acid (Humco, Texarkana, TX). Toddlers remained fasted for the next 1.5 hours before ingesting a second porridge meal. Two weeks post-dosing (D14), a 3 mL venous blood sample was obtained. Approximately three months post-dosing (D84), an additional 5 mL of blood was collected. Following the D84 clinic visit, all participants were treated with mebendazole as previously detailed (16).

Malaria and helminths screening

On D0, D14 and D84, whole blood was screened for malaria parasites using both thick and thin blood films (17). When the D14 blood was obtained, a stool sample was collected for analysis of helminth burden (Ascaris Lumbricoides, Trichuris Trichiura and Hookworm) using

the Kato-Katz method (18). Toddlers that had a positive blood smear for malarial parasites on D0, D14 or D84 were treated for malaria with artusonate/amodaquine.

Hematological and iron status measures

Hb concentrations were measured using a Hemocue (Lake Forest, CA). All iron status indicators were analyzed in both D14 and D84 blood samples, except soluble transferrin receptor (sTfR) which was only analyzed in the D14 samples. Any participant identified as anemic on D14 (Hb < 11.0 g/dL) received liquid iron supplementation (12.5 mg ferrous sulfate/day) (19), from D14 through D84. Serum ferritin (SF) and sTfR were measured by enzyme-linked immunosorbent assay (Ramco Laboratories, Inc Stafford, TX). Total body iron (TBI) was calculated by the ratio of sTfR to SF (TBI (mg/kg) = -[log sTfR*1000/SF) -2.8229]/0.1207) (20,21). Erythropoietin (EPO) and C-reactive protein (CRP) were measured using an Immulite® 1000 Analyzer (Seimens Healthcare Diagnostics, Deerfield, IL). Depleted iron stores were defined when SF concentrations were less than 12 ug/L and CRP concentrations were less than 5 mg/L, or when SF concentrations were less 30 ug/L and CRP concentrations were greater than 5mg/L. Elevated sTfR was classified if concentrations were greater than 8.5 mg/L. Elevated CRP was defined when concentrations were greater than 10 mg/L. Archived serum samples were used to measure hepcidin by a competitive enzyme-linked immunosorbent assay (Intrinsic Life Sciences, La Jolla, CA) on D14 serum (n = 42) and D84 serum (n = 38). Hepcidin values below the limit of detection (5 ng/mL) were assigned a concentration of 2.5 ng/mL.

RBC iron enrichment and incorporation

Iron isotopic ratios were analyzed using magnetic sector thermal ionization mass spectrometry (ThermoScientific, Triton TI, Bremen, FRG). Mass spectrometric methods have been previously described in detail (22,23). The delta percent excess (Δ%xs) of each administered stable iron isotope in RBC was determined by the observed (obs) and natural abundance (NA) isotope ratios:

$$\Delta\%\text{xs } {}^{57}\text{Fe} = \frac{{}^{57}\text{Fe}/{}^{56}\text{Fe}_{\text{obs}} - {}^{57}\text{Fe}/{}^{56}\text{Fe}_{\text{NA}}}{{}^{57}\text{Fe}/{}^{56}\text{Fe}_{\text{NA}}} \times 100$$

A similar equation was used for the 58Fe tracer. Natural abundance ratios used for the 57Fe/56Fe and 58Fe/56Fe ratios were 0.02317686 and 0.0030856, respectively.

To account for the small differences in tracer doses administered, a Dose Adjusted Enrichment (DAE) was calculated for each tracer based on the mean dose of each isotope administered. For those receiving the 57Fe tracer, the mean dose of 57Fe administered was 6.64 mg 57Fe. The 57Fe DAE was calculated as:

$${}^{57}\text{Fe DAE} = \frac{\Delta\%\text{xs } {}^{57}\text{Fe} \times \text{Average Dose of } {}^{57}\text{Fe}}{\text{Actual Dose of } {}^{57}\text{Fe}}$$

A similar equation was utilized to obtain a DAE after calculating the mean dose of 58Fe dose (0.891 mg 58Fe) that would give the same enrichment as that obtained with the average 57Fe dose administered. Throughout the manuscript the term RBC iron DAE reflects this value. In addition to the direct measurements of RBC iron DAE, RBC iron incorporation was calculated in D14 blood as previously reported (22,23). The blood volume in toddlers was estimated using the Linderkamp equation Log (Blood Volume) = 0.6459* (Log Weight in kg) + 0.002743 *(Height in cm) + 2.0324 (24). The change in RBC iron DAE from D14 to D84 post-dosing was determined by subtracting each toddler's D14 DAE from the D84 DAE. Observed decreases in

RBC iron DAE over time may occur due to increases in the size of the circulating RBC pool induced by absorption of natural isotopic iron or mobilization of storage iron (25).

Statistical analysis

Descriptive statistics were used to summarize characteristics of participants, using the mean ± standard deviation (SD) or geometric mean (95% confidence interval (CI)) for continuous variables or frequency counts with percentages for categorical variables. Student's t tests and Wilcoxon's rank-sum tests were used to assess possible differences in concentrations of biochemical indicators between groups stratified by sex, anemia, presence of malarial or helminth infections, or presence of both parasites. Pearson correlation or Spearman rank correlations were used to evaluate the strength of the bivariate linear association between variables. The Chi-squared test of independence was used for analyzing the association between categorical variables. The odds ratio of anemia was evaluated using logistic regression after adjustment for confounders (sex, age, and anthropometric measures). Multiple linear regression was used to estimate the association of RBC iron incorporation and change in RBC iron DAE after controlling for confounding factors such as sex and age. All analyses were performed using the JMP Pro statistical program version 13.1.0 (SAS Institute, Inc., Cary, NC). Significance was defined by a P value less than 0.05.

Results

Characteristics of the study participants

In total, 82 women consented to allow their child to participate in the study and among this group, 71 toddlers completed the study as detailed in **Figure 3.1**.

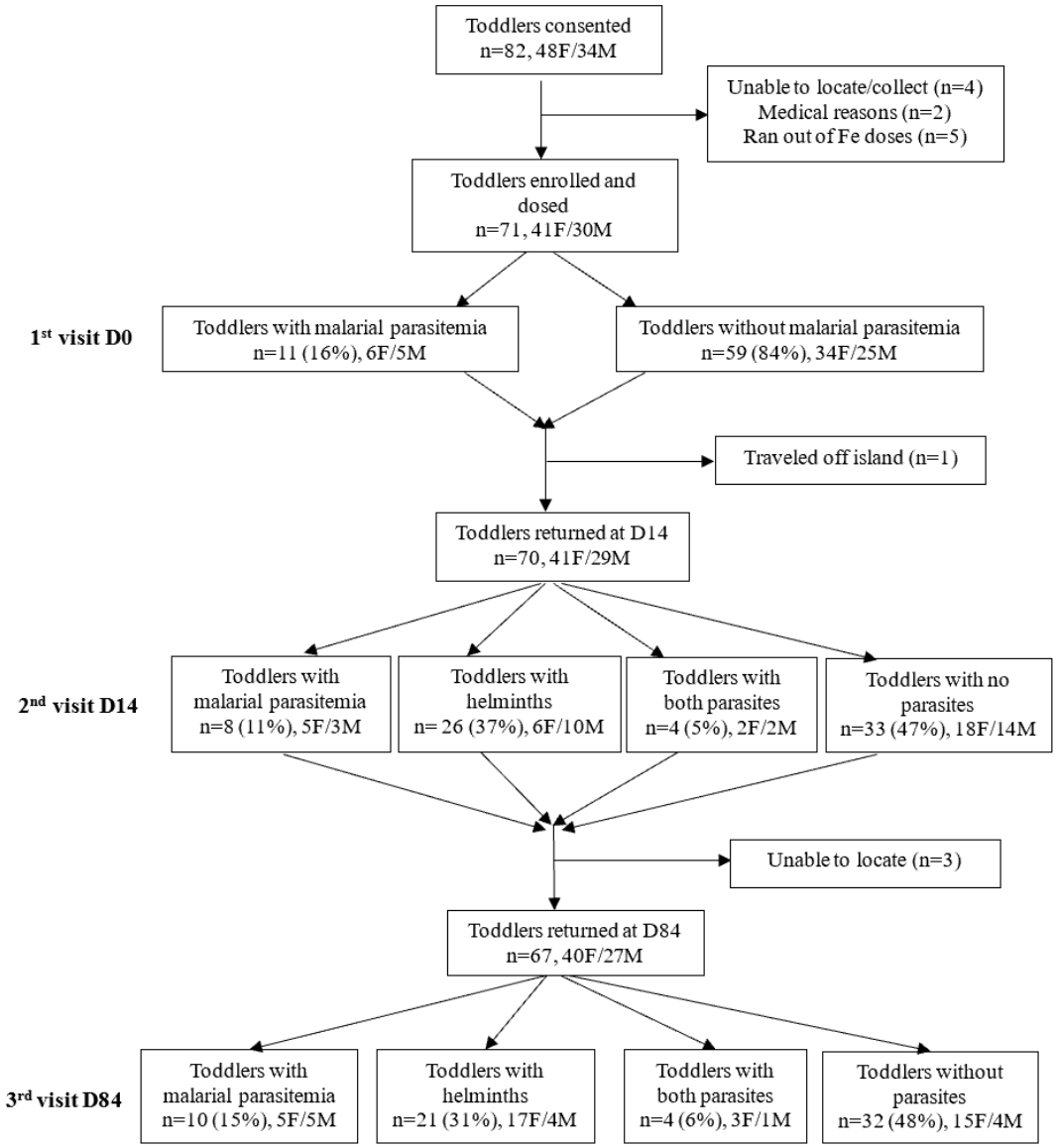

Figure 3.1 Flow chart of toddlers enrolled in the study. Overview of the parasitic infections in Zanzibari toddlers aged 16-25 months. F, Females; M, Males.

Characteristics of participants are reported in **Table 3.1**. More female (n = 41) than male toddlers (n = 30) enrolled in the study. There were no statistically significant differences in the percentage

of stunting or wasting between the males and females, but females tended to be more underweight than the males (P < 0.08). At enrollment on D0, 16% (11/70) of participants had a positive blood smear for malarial parasites. At D14, 37% (26/70) and 11% (8/70) of toddlers had helminths and malarial parasitemia, respectively. At D14, 6% (4/70) of toddlers were infected with both helminths and malaria. Prevalence of malaria and helminth infections did not significantly differ between males and females. On D14, 47% (n = 33) toddlers were anemic and were treated with iron supplementation (iron Supplemented Group). The remaining 53% of children were non-anemic and were not supplemented with iron (Non-iron supplemented Group). Characteristics of these 2 groups of toddlers are presented in **Table 3.2**. The D84 blood sample was collected 84 days after tracer dosing in all but one toddler in whom the blood sample was collected 83 days post-dosing.

Table 3.1 General characteristics of the 71 toddlers enrolled in the study [1]

	All Toddlers (n = 71)	**Females (n = 41)**	**Males (n = 30)**
Age, *month*	19.47 ± 2.19	19.41 ± 2.06	19.55 ± 2.39
Height, cm^2	77.18 ± 4.23	75.91 ± 3.60	78.91 ± 4.48
Weight, kg^3	9.78 ± 1.41	9.13 ± 0.97	10.66 ± 1.45
Weight-for-age z-scores[4]	-0.93 ± 1.06	-1.22 ± 0.93	-0.55 ± 1.13
Underweight, *%*[5]	15.49 [11]	21.95 [9]	6.67 [2]
Length-for-age z-scores	-1.91 ± 1.19	-2.06 ± 1.09	-1.71 ± 1.29
Stunted, *%*	39.43 [28]	41.46 [17]	36.67 [11]
Weight-for-length z-scores	0.00 ± 1.28	-0.26 ± 0.98	0.36 ± 1.55
Wasted, *%*	2.82 [2]	2.44 [1]	3.33 [1]

[1]Data are presented as the mean ± SD or % [n]. Differences between sexes were analyzed by Student's *t* test or Chi-square test.

[2]Significant difference observed between the males and females, $P < 0.005$.

[3]Significant difference observed between the males and females, $P < 0.0001$.

[4]Significant difference observed between the males and females, $P < 0.01$.

[5]Difference between males and females approached significance, $P = 0.08$.

Table 3.2 Iron status in anemic toddlers that received, or did not receive, iron supplementation on D14[1]

	Time-point	Iron Supplemented Group	Non-Iron Supplemented Group
Hb, *g/dL*	D0	10.38 ± 1.41 [33]	11.19 ± 1.42 [35][2]
	D14	9.96 ± 1.02 [33]	12.27 ± 1.00 [36][3]
	D84	11.33 ± 1.30 [32][4]	12.28 ± 1.09 [33][5]
SF, *μg/L*	D14	27.76 (20.54, 37.51) [32]	30.98 ± 19.74 [36]
	D84	34.42 (24.29, 48.78) [32][6]	19.75 (14.77, 26.39) [30][2]
sTfR, *mg/L*	D14	9.03 (7.82, 10.43) [33]	7.76 ± 2.36 [36][2]
EPO, *IU/L*	D14	18.67 (13.66, 25.50) [31]	11.15 (8.76, 14.19) [36][2]
	D84	12.14 (9.18, 16.07) [30][7]	11.49 (8.78, 15.05) [31]
CRP, *mg/L*	D14	0.27 (0.15, 0.49) [31]	0.32 (0.22, 0.48) [36]
	D84	0.37 (0.22, 0.62) [32]	0.28 (0.13, 0.58) [31]
Elevated CRP, %	D14	0	0
	D84	3.13 [1/32]	3.13 [1/32]
Serum hepcidin, *ng/mL*	D14	26.62 ± 21.12 [17]	14.04 (7.56, 26.08) [24]
	D84	48.22 (26.10, 89.08) [16][7]	23.64 (12.02, 46.49) [21]
TBI, *mg/kg*	D14	2.56 ± 3.09 [32]	2.90 ± 2.72 [36]

[1]Data are presented as the mean ± SD [n] or geometric mean (95% CI) [n] or % [n]. Elevated CRP was defined as concentrations greater than 10 mg/L. Differences between toddlers supplemented with iron and without iron supplementation were analyzed by Student's *t* test or Wilcoxon's rank-sum test. Differences between D14 and D84 measurements were analyzed by paired Student's *t* test. CRP, C-reactive protein; D0, first day of the study visit; D14, 14 days post-dosing; D84, 84 days post-dosing; EPO, erythropoietin; Hb, hemoglobin; SF, serum ferritin; sTfR, soluble transferrin receptor; TBI, total body iron.

[2]Significant difference observed between toddlers supplemented with iron and without iron supplementation, $P < 0.05$.

[3]Significant difference observed between toddlers supplemented with iron and without iron supplementation, $P < 0.005$.

[4]Significant change from D14 to D84 observed in toddlers supplemented with iron supplementation, $P < 0.0001$.

[5]Significant difference observed between toddlers supplemented with iron and without iron supplementation, $P < 0.0001$.
[6]A tendency of significant change from D14 to D84 observed in toddlers supplemented with iron supplementation, P = 0.08.
[7]Significant change from D14 to D84 observed in toddlers supplemented with iron supplementation, $P < 0.05$.

Hematological and iron status

On D0, 56% (40/71) of toddlers were anemic. Those with elevated sTfR were nearly 5-fold more likely to be anemic (Odds Ratio: 4.69; 95% CI: 1.37, 13.98). Interestingly, toddlers who had helminth infections had a 40% lower risk of anemia when compared to those without helminth infections. After adjusting for sex, age and anthropometric measurements, anemia was significantly less prevalent in helminth infected toddlers (Adjusted odds ratio (AOR): 0.26; 95% CI: 0.07, 0.97) and significantly more prevalent among those with elevated sTfR (AOR: 4.74; 95% CI: 1.01, 22.15).

As expected, mean Hb concentration increased from D14 to D84 in those who were iron supplemented, but no significant change in Hb was observed in the non-iron supplemented group (P = 0.65) (**Table 3.2**). In addition, iron supplemented toddlers had a significant decrease in EPO from D14 to D84 as well as a tendency for SF concentrations to increase (**Table 3.2**). There were no significant changes in EPO or SF in the non-iron supplemented group.

Hepcidin analyses were only conducted in a sub-group of toddlers (n = 41 at D14 and n = 37 at D84) based on availability of serum. Hepcidin concentrations on both D14 and D84 did not significantly differ between the anemic and non-anemic participants. Hepcidin concentrations, however, did increase significantly from D14 to D84 in the iron supplemented group but not among the non-iron supplemented group (P = 0.32) (**Table 3.2**). At D14, hepcidin was inversely

correlated with D14 sTfR ($P < 0.05$, $r = -0.42$) and positively correlated with D14 SF ($P < 0.05$, $r = 0.34$). There was a lack of correlation between hepcidin and CRP on D14. At D84, hepcidin concentrations remained significantly correlated with D84 SF concentrations ($P = 0.005$, $r = 0.42$) and were also positively correlated with D84 CRP values ($P < 0.05$, $r = 0.49$).

Participants with malarial parasitemia on D0 had impaired iron and hematological status as evident by significantly lower D14 TBI, higher D14 sTfR and D14 EPO concentrations and a tendency for lower D0 Hb concentrations (**Table 3.3**). The iron status of toddlers with malarial parasitemia on D14 is presented in **Table 3.3**. The iron status of those with helminths did not differ from those without helminths at D14. For the subset of toddlers who had hepcidin measured, D14 hepcidin was significantly lower in those who had helminth infections ($P = 0.03$, $n = 42$) (data not shown).

Table 3.3 Iron status as a function of malaria parasitemia[1]

	Time-point	Malaria Parasitemia at D0		Malaria Parasitemia at D14	
		Yes	**No**	**Yes**	**No**
Hb, *g/dL*	D0	9.98 ± 1.46 [11]	11.00 ± 1.42 [58][2]	10.19 ± 1.74 [8]	10.91 ± 1.42 [61]
	D14	11.00 ± 1.00 [11]	11.17 ± 1.60 [58]	10.74 ± 2.07 [8]	11.23 ± 1.45 [62]
	D84	12.20 ± 0.91 [11]	11.70 ± 1.32 [55]	11.66 ± 0.92 [8]	11.85 ± 1.33 [58]
SF, *µg/L*	D14	21.5 (14.4, 32.3) [11]	29.3 (23.0, 37.5) [57]	41.4 (28.1, 61.1) [8]	26.3 (20.9, 33.2) [61][3]
	D84	20.9 (11.7, 37.5) [11]	28.4 (22.1, 36.6) [52]	24.7 (13.4, 45.3) [8]	26.6 (20.7, 34.5) [55]
sTfR, *mg/L*	D14	10.49 (9.12, 12.06) [11]	7.77 (7.03, 8.55) [58][4]	9.28 (7.27, 11.85) [8]	7.97 (7.26, 8.76) [62]
EPO, *IU/L*	D14	21.97 (13.90, 34.71) [11]	13.20 (10.59, 16.34) [56][4]	25.51 (13.94, 46.68) [7]	13.20 (10.70, 16.20) [61][3]
	D84	17.77 (11.72, 26.96) [11]	10.99 (8.94, 13.46) [51][4]	16.09 (8.86, 29.25) [8]	11.25 (9.21, 13.74) [54]
CRP, *mg/L*	D14	0.44 (0.20, 0.97) [11]	0.28 (0.19, 0.41) [56]	0.87 (0.27, 2.83) [7]	0.28 (0.19, 0.39) [61]
	D84	0.35 (0.11, 1.17) [11]	0.32 (0.20, 0.51) [53]	1.55 (0.39, 6.23) [8]	0.25 (0.16, 0.39) [56][3]
Elevated CRP, %	D14	0	0	0	0
	D84	9.09 [1/11]	1.89 [1/53]	12.5 [1/8]	1.79 [1/56]
Serum hepcidin, *ng/mL*	D14	11.88 (4.31, 32.75) [9]	17.37 (10.58, 28.49) [32]	24.85 (16.36, 37.75) [6]	14.15 (8.61, 23.18) [36]
	D84	14.95 (5.93, 37.67) [9]	44.70 (27.54, 72.68) [28][4]	53.25 (8.69, 327.01) [6]	29.07 (18.23,46.35) [32]
TBI, *mg/kg*	D14	1.12 ± 2.54 [11]	3.52 {4.16} [57][4]	3.28 {2.64} [8]	3.37 {4.13} [61]

[1]Data are presented as the mean ± SD [n] or geometric mean (95% CI) [n] or median {interquartile range} [n] or % [n]. Elevated CRP was defined as concentrations greater than 10 mg/L. Differences between toddlers with and without malaria parasitemia at D0 or D14 were analyzed by Student's *t* test or Wilcoxon's rank-sum test. CRP, C-reactive protein; D0, first day of the study visit; D14, 14 days post-dosing; D84, 84 days post-dosing; EPO, erythropoietin; Hb, hemoglobin; SF, serum ferritin; sTfR, soluble transferrin receptor; TBI, total body iron.
[2]Difference between toddlers with and without malaria parasitemia at D0 approached significance, $P = 0.05$.
[3]Significant difference observed between toddlers with and without malaria parasitemia at D14, $P < 0.05$.
[4]Significant difference observed between toddlers with and without malaria parasitemia at D0, $P < 0.05$.

RBC iron incorporation

Iron incorporation data are presented in **Table 3.4**. RBC iron incorporation at D14 was highly variable (ranging from 1.21 - 55.45%). As expected, average RBC iron incorporation did not significantly differ between those that received the 58Fe or the 57Fe tracer. TBI was correlated with RBC iron incorporation (P = 0.009, r = -0.32, n = 69) but there were no other significant associations with other iron status indicators or with hepcidin. Infection with both parasites was associated with significantly higher RBC iron incorporation, whereas infection with helminths only tended to be associated with higher RBC iron incorporation (P = 0.07). Toddlers with malarial parasitemia did not have higher RBC iron incorporation compared to those without this infection (**Table 3.4**). The association between iron incorporation and parasitic infections did not appear to be driven by low iron status as there were no significant associations between presence of either or both parasitic infections with any of the iron status indicators measured. Mean D14 hepcidin tended to be higher (P = 0.07) in those without malaria and/or helminth infections compared to the mean observed in those with either or both of these parasitic infections (10.24 ng/mL). In the subgroup of participants with either malarial or helminth infection, RBC iron incorporation was negatively correlated with CRP (P < 0.05, r = -0.38, n =

31) but was not significantly impacted by hepcidin on D14. RBC iron incorporation did not differ between the iron supplemented or non-supplemented toddlers (P = 0.5). Significantly higher RBC iron incorporation was evident among those with higher weight-for-age z-scores, lower TBI, and among those with helminth infections (P < 0.05). Hepcidin was not significantly associated with RBC iron incorporation, which may be due to the high variability in hepcidin coupled with the known impact of infection on the concentrations of this biomarker.

Table 3.4 Factors associated with red blood cell iron incorporation (%) at D14[1]

Groups	**Iron incorporation at D14 (%)**	***P***
All toddlers	11.42 (9.29, 14.02) [68]	**---**
Females	10.89 (8.44,14.05) [40]	NS
Males	12.22 (8.52, 17.53) [28]	
D14 Anemia	11.41 (8.54, 15.25) [33]	NS
No D14 Anemia	11.42 (8.41, 15.52) [35]	
D14 Helminths	14.46 (10.54, 19.82) [26]	0.07
No D14 Helminths	9.87 (7.53, 12.92) [42]	
D14 Malaria parasitemia	10.03 (4.44, 22.68) [8]	NS
No D14 Malaria parasitemia	11.61 (9.35, 14.43) [60]	
Presence of parasites	14.50 (10.97, 19.15) [31]	0.03
No parasites	9.17 (6.80, 12.37) [36]	
D14 Detectable hepcidin	8.55 (6.16,11.87) [28]	0.08
D14 Undetectable hepcidin	14.76 (8.63, 25.27) [12]	

[1]Data are presented as the geometric mean (95% CI) [n]. Comparison groups are stratified by sex, presence of anemia on D14, presence of malarial or helminth infections on D14 or either parasites over the study period, or by hepcidin concentrations on D14. Hepcidin values below 5 ng/mL were defined as undetectable hepcidin. Differences were analyzed by Student's *t* test or Wilcoxon's rank-sum test. D14, 14 days post-dosing; NS, not significant.

Change in RBC iron DAE from D14 to D84

RBC iron DAE decreased from D14 to D84 on average by 2.75 percentage-points (from 15.94% to 13.19%; 95% CI: -3.86 percentage-points, -1.65 percentage-points, $P < 0.0001$, $n = 66$), but the degree of change varied more than 6-fold from -16.41 percentage-points to +12.75 percentage-points. Among the 66 toddlers with data available on the change in RBC iron DAE between D14 and D84, 12 toddlers (28%) exhibited slight increases in RBC iron DAE from D14 to D84. Those with increases in RBC iron DAE were equally distributed among toddlers that had received the 57Fe ($n = 5$) or 58Fe ($n = 7$) tracer. On average, in these 12 individuals, the mean increase observed was 1.67 ± 3.52 percentage points. After excluding the toddler with the highest increase (+12.75 percentage points), the mean RBC iron DAE increase observed was 0.66 ± 0.49 percentage points. Possible differences in those that exhibited increases in DAE over time versus those that exhibited decreases in DAE over time were explored. Toddlers that had undetectable hepcidin on D84 ($P = 0.02$) and toddlers with lower sTfR concentrations on D14 ($P = 0.04$) were significantly more likely to have had an increase in RBC iron DAE between D14 to D84. In addition, participants with lower RBC iron DAE at D14 were more likely ($P = 0.04$) to experience increased RBC iron DAE at D84. These results remained significant even when data from the toddler with the largest 12.75 percentage points increase were excluded.

The net change in RBC iron DAE over time was not significantly impacted by sex, age, anthropometric measurements or malarial infections. A comparison of the change in RBC iron DAE between different groups is presented in **Figure 3.2**.

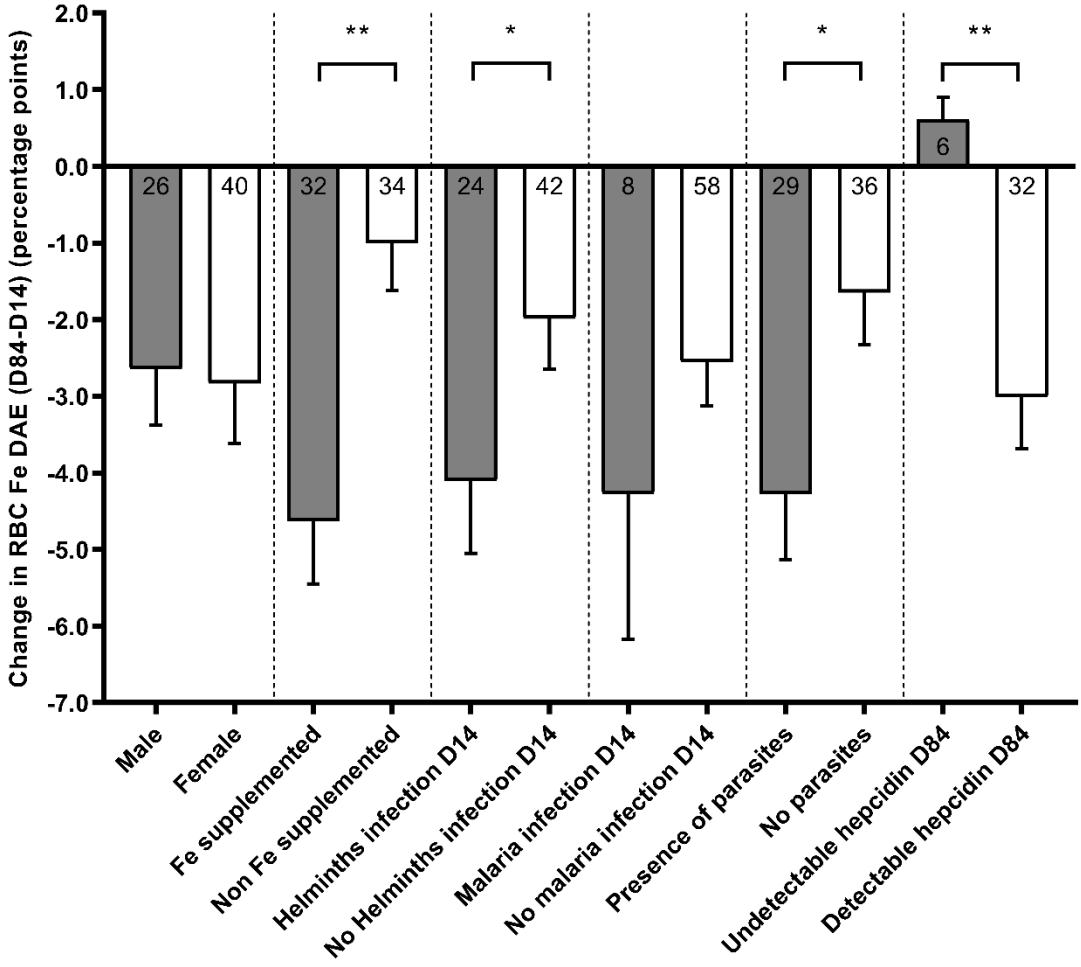

Figure 3.2 Mean change in RBC iron DAE (D84-D14) in toddlers as a function of sex, iron status or parasitic burden. Comparison groups are stratified by sex, iron supplementation on D14, presence of malarial or helminth infections on D14 or either parasites over the study period, or by hepcidin concentrations on D84. Hepcidin values below 5 ng/mL were defined as undetectable hepcidin. Number on each bar indicates the number of toddlers in each group. Differences were analyzed between each pair of groups by Wilcoxon's rank-sum test, $^{*}P < 0.05$, $^{**}P < 0.005$. Error bars represent SEMs. DAE, Dose Adjusted Enrichment; D14, 14 days post-dosing; D84, 84 days post-dosing; RBC, red blood cell.

Change in RBC iron DAE was significantly greater in those who were supplemented with iron (P = 0.001) versus those who had not been iron supplemented. This change in DAE in those receiving iron supplementation appeared to be due to the addition of iron and not a consequence

of the pre-existing anemia in this group, based on the finding that toddlers with anemia at D0 did not have a significantly greater change in RBC iron DAE compared to non-anemic toddlers at D0 (data not shown). Participants with higher hepcidin concentrations on D84 had greater reductions in RBC iron DAE compared to those with undetectable hepcidin at D84 ($P = 0.003$, $n = 38$). RBC iron DAE decreased to a larger extent in those found to be infected with either malaria or helminths ($P = 0.02$) across the study period. Toddlers with helminths on D14 had greater losses in RBC iron DAE over the 70-day observation period ($P = 0.05$) but the presence of malarial infections did not have a significant impact on change in RBC iron DAE over time ($P = 0.19$) perhaps due to the limited sample size of those with malaria ($n = 8$). Among all hematological and iron status indicators analyzed, Hb (either at D0 or D14) was the only biomarker that was significantly negatively associated with change in RBC iron DAE over time ($P < 0.005$). In addition, an increase in Hb from D14 to D84 was significantly correlated with greater decrease in RBC iron DAE from D14 to D84 ($P < 0.005$, $r = 0.37$).

A multiple linear regression model was constructed to identify factors significantly associated with the change in RBC iron DAE. On D14, iron supplementation ($\beta \pm$ SE (standard error) $= 0.40 \pm 0.47$; $P = 0.0005$), RBC iron incorporation ($\beta \pm SE = 0.44 \pm 0.04$; $P = 0.0006$) and helminth infection ($\beta \pm SE = 1.36 \pm 0.54$; $P = 0.03$) were found to be significantly associated with a decrease in RBC iron DAE from D14 to D84 (Adjusted $R^2 = 0.38$; $n = 62$). A second model was constructed in the sub-group of toddlers with hepcidin data (Adjusted $R^2 = 0.54$; $n = 36$) setting the change in RBC iron DAE as the dependent variable and iron incorporation and iron status indicators as independent variables. In this smaller sub-population, helminth infection was no longer significantly associated with change in RBC iron DAE but those with detectable

hepcidin at D84 exhibited a significantly greater loss in RBC iron DAE ($\beta \pm SE = 0.30 \pm 0.62$; $P = 0.03$).

Discussion

A better understanding of the impact of parasitic infections on iron metabolism is needed, especially if iron supplementation may exacerbate adverse events when given to iron replete children with active parasitic infections. To our knowledge, this study is one of the largest iron stable isotope studies to follow RBC iron DAE in this age group over an 84-day period. Toddlers with better weight-for-age z-scores, lower TBI and those with helminth infections exhibited higher RBC iron incorporation. A greater decrease in RBC iron DAE post-dosing was evident among those receiving iron and among those with helminth infections.

An unexpected finding was that toddlers who had helminth infections had a nearly 40% lower risk of anemia compared to those without helminth infections. An earlier study in a larger cohort of Zanzibari children in the parent study (13) also found a lower prevalence of anemia in children (6-23 months) with helminth infections compared to children without helminth infections. In contrast, in older Zanzibari children (7-13 years), Stoltzfus et al. (26) found that hookworm infection was the strongest risk factor for severe anemia. Age differences between studies may in part explain these differences as anemic and poorly nourished toddlers may not be actively exploring their environments and thus may have less exposure to helminth-infected soil.

Toddlers that experienced either malarial or helminth infection across the study had significantly higher RBC iron incorporation than those who remained parasite-free, an effect that appeared to be driven primarily by the non-significant increase in RBC iron incorporation in those with helminth infections. RBC iron incorporation did not differ between toddlers with and without malaria parasitemia but the small number with malaria parasitemia limited our ability to

address the independent effect of malaria on study outcomes. Participants in our study incorporated an average of 11.4% of ingested iron into their RBCs. This is comparable to the 9.0% and 11.5% RBC iron incorporation found among 48 Malawian toddlers (12–24 months) that received iron supplements (30 mg/day) either immediately, or two weeks after, being treated for malaria (27). After 2 weeks of oral iron supplementation, mean SF concentration increased by 2.5-fold and iron incorporation decreased significantly to 5.9% in the Malawian cohort (27). This result is consistent with our finding of a lower RBC iron incorporation in those with better iron status. Average RBC iron incorporation in our study was slightly lower than the 18% reported in 27 Gambian toddlers (18–36 months) who were dosed with a 43% lower quantity of iron tracer (4 mg) (21).

Among all biomarkers evaluated, the strongest determinant of RBC iron incorporation was TBI. Our finding that lower iron status, but not decreased inflammation, explained more variability in RBC iron incorporation is in agreement with prior data (28). It is likely that TBI better captures iron demands by accounting for both tissue demand and storage iron, and TBI may attenuate the impact of inflammation in populations at risk for recurrent parasitic infections. Data from 156 Ugandan children (1.5–5 years) found that subclinical inflammation (evidenced by elevated hepcidin and CRP) persisted for 12 months after antimalarial treatment (11,29). In our cohort, hepcidin was not a significant determinant of RBC iron incorporation. This finding differs from that observed in a population of anemic Gambian toddlers (21), where hepcidin was found to be the best predictor of RBC iron incorporation. When toddlers are actively inflamed, hepcidin may explain more variability in RBC iron incorporation whereas iron status may be a better predictor of RBC iron incorporation in the absence of inflammation. We were unable to obtain measures of hepcidin on D0. It is likely that in this population with frequent infections,

the D14 hepcidin measure may not have adequately captured hepcidin status at the time tracer was administered and absorbed. Our data also indicate that helminth infections are associated with higher iron incorporation, which has also been reported in Ivorian children (30).

This study uniquely followed the change in RBC iron isotopic enrichment over 84-days in healthy toddlers residing in a malaria- and helminth-endemic area. On average RBC iron DAE decreased over the 84-days, but a small subset of our cohort exhibited a slight increase in RBC iron DAE over time. Those that exhibited increases in RBC iron DAE were significantly more likely to have undetectable hepcidin at D84, and lower sTfR concentrations and RBC iron DAE at D14. The slight increase in RBC iron DAE observed draws attention to the need to further refine the timing used to evaluate RBC iron incorporation in healthy toddlers and/or in those with parasitic infections. This methodology and the measure of RBC incorporation 2-week after dosing was developed from early radiotracer data in adults (31). There are no other data evaluating changes in RBC iron enrichment over time in toddlers to determine if our findings are unique to our cohort, or if the timing of RBC iron incorporation is more variable in this age group. Thus, further normative data on this methodology in children are warranted.

In these toddlers, decreases in RBC iron DAE were most pronounced in those who received iron supplementation and exhibited significant increases in Hb post-supplementation. Greater losses in RBC iron DAE over time did not appear to be driven by pre-existing anemia as the D14 to D84 decline in RBC iron DAE was not significantly impacted by the presence of anemia at D0. In addition, iron supplementation was a stronger determinant of change in RBC iron DAE than was the presence of malaria and/or helminth parasites. Two other studies in toddlers followed RBC iron enrichment over an extended period. Fomon et al. (32) studied 35 Caucasian infants (5.6 months) and found the change in RBC iron enrichment over 13 months

was significantly correlated with iron absorption, which is similar to our finding of greater decreases in RBC iron DAE among toddlers with higher RBC iron incorporation. More recently, Speich et al. (25) dosed a group of African toddlers (14-20 months) and allowed for isotopic equilibration to occur for an 8-11 month period post-dosing before evaluating the impact of iron supplementation on RBC tracer enrichment. Our study cannot be directly compared to the Speich study as we did not allow sufficient time for tracer to equilibrate with body iron pools but there are some similarities in findings. Consistent with their study, we found that hepcidin was not significantly correlated with RBC iron incorporation and observed decreases in RBC iron enrichment were positively correlated with iron absorption, increases in Hb and iron supplementation. They uniquely found that although iron supplementation increased the absolute amount of iron absorbed the additional iron absorbed did not appear to be retained (25).

Our study contributes to the data available on factors that impact RBC iron incorporation and change in RBC iron DAE over time in toddlers residing in a malaria- and helminth-endemic area but there are several limitations. Inflammatory status was evaluated using CRP, but this may not be a robust inflammatory marker in those with chronic infections (33–35). We could not differentiate a new onset of malarial infection from a relapse of an existing infection. Although anti-malarial/helminths medications were provided, recurrence of new infections was difficult to prevent in a parasite-endemic context. Our classification of infection as one that involved either malaria or helminths likely increased our power to identify effects of these two parasitic infections, we could not fully evaluate the impact of each individual infection due in particular to the lower number of toddlers with malaria.

In summary, RBC iron incorporation was best predicted by weight-for-age z-scores and TBI in these Zanzibari toddlers. RBC iron DAE decreased significantly across an interval of time

that approaches the typical RBC lifespan in this age group (36). Toddlers who received iron, incorporated more iron into RBC's exhibited significantly greater losses in RBC iron DAE post-dosing. Future longer-term labeling studies may provide greater insight into whole body iron homeostasis and adaptations to parasitic loads or iron supplementation in these vulnerable age groups.

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CHAPTER 4

ETHNIC DIFFERENCES IN IRON STATUS*

* Wanhui Kang, Alexa Barad, Andrew G. Clark, Yiqin Wang, Zhenglong Gu, Kimberly O. O'Brien. Ethnic differences in iron status. *Advances in Nutrition*, 2021;00:1–16; doi:https://doi.org/10.1093/advances/nmab035.

Abstract

Iron is unique among all minerals in that humans have no regulatable excretory pathway to eliminate excess iron after it is absorbed. Iron deficiency anemia occurs when absorbed iron is not sufficient to meet body iron demands, whereas iron overload and subsequent deposition of iron in key organs occurs when absorbed iron exceeds body iron demands. Over time, iron accumulation in the body can increase risk of chronic diseases, including cirrhosis, diabetes, and heart failure. To date, only ~30% of the interindividual variability in iron absorption can be captured by iron status biomarkers or iron regulatory hormones. Much of the regulation of iron absorption may be under genetic control, but these pathways have yet to be fully elucidated. Genome-wide and candidate gene association studies have identified several genetic variants that are associated with variations in iron status, but the majority of these data were generated in European populations. The purpose of this review is to summarize genetic variants that have been associated with alterations in iron status and to highlight the influence of ethnicity on the risk of iron deficiency or overload. Using extant data in the literature, linear mixed-effects models were constructed to explore ethnic differences in iron status biomarkers. This approach found East Asians had significantly higher concentrations of iron status indicators (serum ferritin, transferrin saturation and hemoglobin) compared to Europeans, African Americans, or South Asians. African Americans exhibited significantly lower hemoglobin concentrations compared to other ethnic groups. Further studies of the genetic basis for ethnic differences in iron metabolism and on how it affects disease susceptibility among different ethnic groups are needed to inform population-specific recommendations and personalized nutrition interventions for iron-related disorders.

Introduction

Iron is an essential trace element involved in numerous metabolic processes, including oxygen transport and utilization, cellular proliferation, DNA synthesis, neurotransmitter synthesis, and energy production (1,2). Iron is the fourth most common element in the Earth's crust (1,3), yet iron deficiency (ID) remains prevalent, affecting an estimated 2.5 billion people worldwide (4). Iron overload (IO), on the other hand, is associated with adverse health outcomes caused by the accumulation of iron in organs particularly the pancreas, liver and heart. Elevated iron stores are associated with increased risk of type 2 diabetes independent of inflammation (5,6), and increased risk of cardiovascular disease (7,8), liver fibrosis, and cancer (9–11). Moreover, age-related iron accumulation in the brain strongly predicts cognitive decline, motor impairment, and the development of neurodegenerative diseases (12,13). A causal role of enhanced iron status in these diseases has been noted in animal models of IO (14,15).

Increased attention has been focused on the genetic contributions to iron status. Population differences in the frequency of genetic variants that are associated with increased risk of iron disorders may explain varying iron status in different ethnic groups. No studies to date have pooled existing data to summarize current findings and knowledge on ethnic differences in iron status. This review summarizes data on genetic variants found to be associated with iron metabolism across different ethnic groups and statistically evaluates published data on iron status as a function of ethnicity.

Current Status of Knowledge

Iron physiology

Iron absorption

Dietary iron is ingested as heme iron (from animal-based foods) and non-heme iron (from animal- and plant-based foods). Heme iron is only minimally impacted by iron stores. The proteins involved in heme iron absorption are unique to heme iron and the pathways involved in this process continue to be elucidated (16–18). In contrast, non-heme iron absorption is tightly regulated in response to body iron demands and mutations in the proteins involved in non-heme iron absorption are associated with known iron-related diseases (16,19,20).

Dietary non-heme iron is ingested primarily as ferric iron (Fe^{3+}) that must be reduced to ferrous iron (Fe^{2+}) (by duodenal cytochrome b) before it enters the enterocyte via the divalent metal transporter 1 (DMT1). Once absorbed, Fe^{2+} can either be stored as ferritin, used for intracellular functions, or exported via the only known non-heme iron export protein in the body, ferroportin (FPN). Once exported, the ferroxidase hephaestin oxidizes Fe^{2+} to Fe^{3+}, which can then bind to transferrin (TF) in the bloodstream (16,20,21). At other locations in the body, ceruloplasmin functions as a ferroxidase (22), and the placenta also expresses a unique ferroxidase (zyklopen) (23).

Systemic iron homeostasis

Transferrin-bound Fe^{3+} in circulation is taken up by cells using receptor mediated endocytosis via transferrin receptor 1 (TFR1). Cellular expression of TFR1 is highest in erythroid tissue to support erythropoiesis. Under normal circumstances, approximately 30% of the iron-binding sites in the plasma TF pool are occupied. This value is referred to as transferrin

saturation (TSAT) (16,24). When TSAT exceeds 45%, iron begins to circulate free or bound to low molecular weight molecules (citrate, albumin) generating potentially toxic iron species known as non-transferrin bound iron (NTBI). The pancreatic cells and hepatocytes can internalize NTBI via Zrt- and Irt-like protein 14, while cardiomyocytes are purported to internalize NTBI through L-type or T-type calcium channels (25,26). Cellular uptake of NTBI can increase the intracellular labile iron pool resulting in generation of reactive oxygen species that can cause oxidative damage, adversely impacting specific organs and over time, increasing the risk of chronic diseases.

Only 1-2 mg/day of absorbed iron is needed to offset the typical amounts of endogenous daily iron losses. The majority of iron utilized to support erythropoiesis (20-25 mg/day) is obtained from catabolism of senescent RBCs. Several hormones are now known to be involved in the regulation of systemic iron homeostasis including hepcidin, erythropoietin, and erythroferrone.

Hormonal control of iron physiology

Of the three iron-related hormones, hepcidin is the major regulator of body iron balance. This hepatic hormone binds to FPN thereby reducing iron export from the enterocyte and iron release from stores (27). Hepcidin production is induced by iron loading or inflammation/infection and suppressed by increased erythropoietic demand or hypoxia. The regulation of hepcidin involves multiple membrane proteins (MT-2, matriptase-2; HJV, hemojuvelin; BMPR 1 and 2, bone morphogenetic protein receptor 1 and 2; TFR1 and 2; HFE, high iron protein; IL-6 receptor), intracellular signaling pathways (BMP-SMAD, BMP-small mothers against decapentaplegic; JAK-STAT, janus kinase-signal transducer and activator of

transcription protein), and hormones (erythropoietin, erythroferrone) (21,28–30). Genetic mutations in these proteins are associated with iron disorders as shown in **Figure 4.1**.

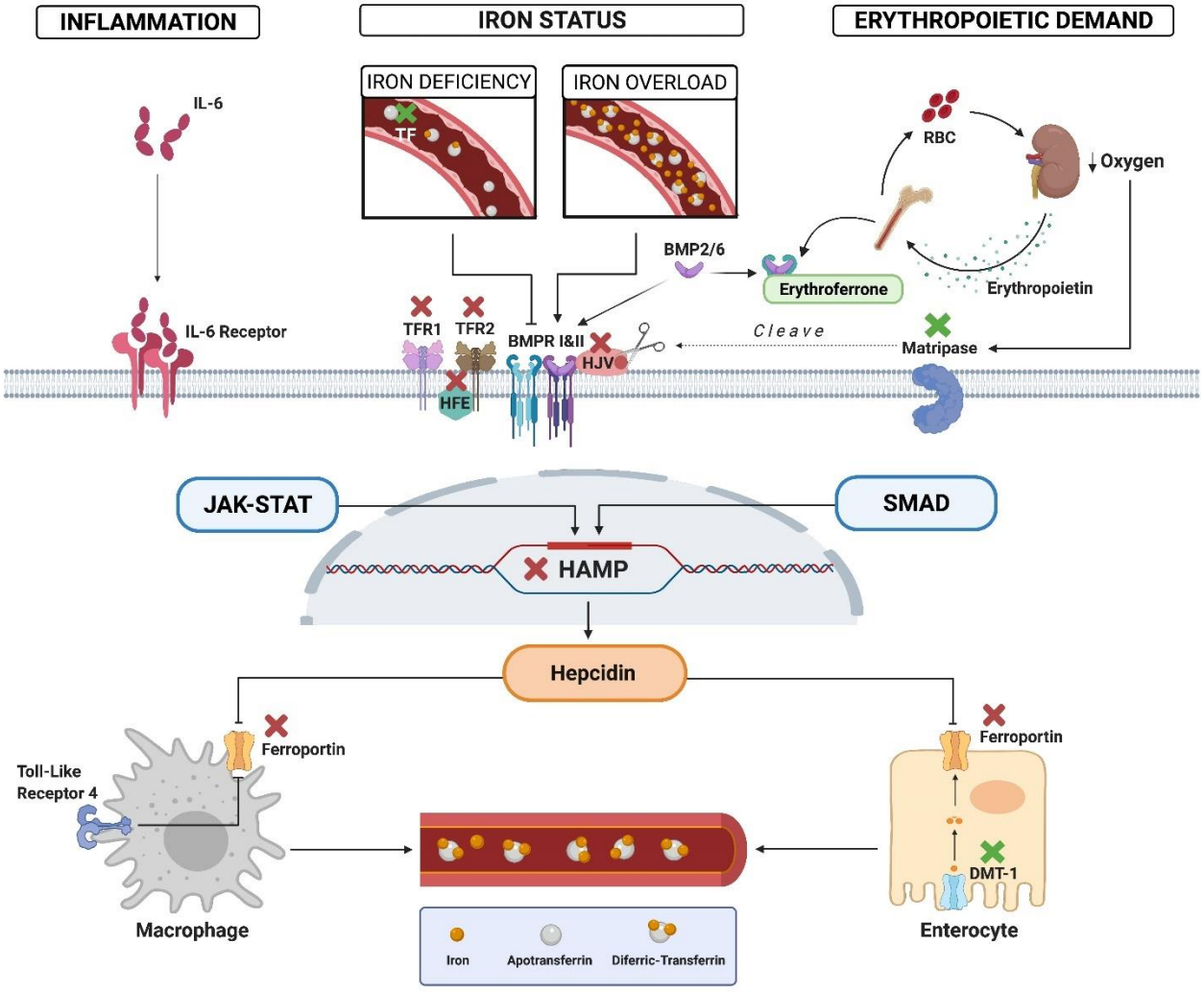

Figure 4.1 The hepatic hepcidin signaling pathway and common genetic mutations in this signaling pathway. Hepcidin is regulated by infection/inflammation (left), iron status (middle), and erythropoietic demand (right). Pathogenic mutations in genes that encode proteins involved in the regulation of the hepcidin or FPN result in iron deficiency (green X) or iron overload (red X). When serum transferrin is saturated, diferric-transferrin binds to TFR1 and displaces HFE. HFE can then form a complex with TFR2 and possibly HJV to promote the BMP-SMAD hepcidin signaling pathway. Recessive mutations in the genes encoding these proteins (HFE, TFR2, HJV, hepcidin) result in decreased hepcidin production preventing hepcidin from being upregulated as iron stores accumulate leading to iron overload. Under low oxygen conditions, MT-2 cleaves HJV, generating a soluble and inactive form of this protein, resulting in the inactivation of the BMP-SMAD signaling pathway and downregulation of hepcidin transcription. Genetic mutations in MT-2 result in uninhibited hepcidin production leading to IRIDA. High

erythropoietic demand results in upregulation of erythroferrone, which suppresses hepcidin production by sequestering BMP6 and inhibiting BMP-SMAD signaling. In inflammation, IL-6 binds to IL-6 receptor, stimulating the JAK-STAT pathway and upregulating hepcidin production. Dominant gain- and loss-of-function FPN1 mutations result in hepcidin resistance or in cellular iron accumulation (particularly in macrophages), respectively. Recessive mutations in DMT1 result in iron loading anemia. Likewise, recessive mutations in TF result in reduced concentrations of functional TF and iron loading anemia. The figure was created using BioRender.com. BMP, bone morphogenetic protein; BMPR, bone morphogenetic protein receptor; DMT-1, divalent metal transporter 1; FPN, ferroportin; HAMP, hepcidin, HFE, high iron protein, HJV, hemojuvelin; IRIDA, iron refractory iron deficiency anemia; JAK-STAT, Janus kinase-signal transducer and activator of transcription protein; MT-2, matriptase-2; SMAD, small mothers against decapentaplegic; TF, transferrin; TFR, transferrin receptor.

Assessment of iron status

Multiple biomarkers are used to represent iron status of individuals and/or populations. Ferritin is the primary intracellular iron storage protein and some is released into circulation as serum ferritin (SF) (31). Low SF concentrations are indicative of depleted iron stores. Elevated concentrations of SF can indicate excessive iron stores or inflammatory conditions since ferritin is an acute phase protein (31). Total iron binding capacity (TIBC) indicates the total number of binding sites for iron in TF, which is the main iron transport protein in circulation. TIBC, in combination with serum iron, can be used to calculate TSAT, which reflects iron supply to tissues (31). Soluble transferrin receptor (sTfR) reflects the intensity of erythropoietic and cellular demands for iron (31). An increase in sTfR concentrations indicates an increase in cellular iron requirements or insufficient iron supply to tissues (31). In late stages of ID, hemoglobin production is compromised which can result in anemia (31). A summary of the typical cutoffs for ID and/or IO for common iron status indicators is provided in **Appendix Table 1.**

Genetic variants of iron metabolism

Tight control of iron absorption is needed to prevent ID or IO but to date, only about 30% of the interindividual variability in iron absorption can be captured by hepcidin or other iron status biomarkers (32–36). Marked ethnic differences in the risk of ID or IO have been known to exist as highlighted by the CDC cut-offs used to denote anemia (37). To investigate the genetic contributions to variations in iron status between and within populations, multiple genome-wide association studies (GWAS) and candidate gene association studies have been undertaken. The following sections cover pathogenic mutations in iron-related genes identified to date that result in ID or IO, and polymorphisms within iron- and non-iron related genes found to be associated with variations in iron traits among healthy populations. Genetic variants associated with iron status in healthy populations stratified by ethnic group are summarized (**Appendix Table 2**).

Genetic variants related to iron overload

Genetic mutations in proteins involved in the regulation of the hepcidin/FPN axis result in aberrant expression of hepcidin or FPN (38) and can eventually lead to IO. Disorders of the hepcidin/FPN axis cause subtypes of hereditary hemochromatosis (HH), a heterogeneous genetic condition found primarily in Northern Europeans. The most common form of HH occurs from mutations in *HFE*, but HH can also result from mutations in *HJV*, hepcidin gene (*HAMP*), *TFR2*, and *FPN1*, which are collectively referred to as non-*HFE* hemochromatosis. The pattern of inheritance of pathogenic mutations in *HFE, HJV, HAMP* and *TFR2* is autosomal recessive, whereas in *FPN1* is autosomal dominant (39). While mutations in these iron-related genes have

been described as causative for HH, some single nucleotide polymorphisms (SNP) within, or in close-proximity to these genes, have been associated with iron traits in healthy populations.

HFE

HFE was named as the high iron (high Fe) or hemochromatosis protein when it was discovered in 1996 to harbor a mutation that was highly prevalent in patients with hemochromatosis (40). This major histocompatibility complex class I like protein stimulates the intracellular BMP-SMAD hepcidin signaling pathway. Recessive mutations in *HFE* result in decreased hepcidin production and thus, hepcidin is not appropriately up-regulated as iron stores accumulate (41) (**Figure 4.1**). *HFE*-related hemochromatosis or HH type 1 is most commonly associated with two missense mutations, C282Y and H63D. The iron burden and clinical phenotype presented in patients with *HFE*-related hemochromatosis is highly variable. The classic biochemical abnormalities seen in C282Y homozygous individuals include elevated TSAT and SF, and tissue IO. Although biochemical penetrance of homozygosity for this mutation is high (75-100% in males, 40-60% in females) (42–44), the clinical penetrance is much lower, affecting men at higher rates than women (2-38% and 1-10%, respectively) (45,46). Interestingly, H63D homozygosity rarely results in clinical disease development, except when this mutation is present with C282Y, both in heterozygote states (40,47).

The prevalence of the C282Y and H63D mutations varies among ethnic groups and is one of the best examples of ethnic differences in iron metabolism (48). The C282Y homozygosity is most prevalent (0.3-0.5%) in individuals of Northern European descent (44,49–52). This mutation is thought to have originated in a Celtic population 60-70 generations ago (53–55). A Viking origin of this mutation has been proposed as highest frequencies are observed in populations of Northern European descent (*i.e.* Viking populations) (56). The Hemochromatosis

and Iron Overload Screening (HEIRS) study reported the highest prevalence (0.44%) of C282Y homozygosity among unrelated non-Hispanic white individuals (44). The prevalence of C282Y homozygosity has been reported at even higher frequencies in specific regions of Northern Europe, particularly Ireland (1.24-1.96%) (57,58). Additionally, the highest average allele frequency in *HFE* has been reported among this population (10.1%) (48). The lowest prevalence of C282Y homozygosity in the HEIRS study was reported among Asians (0.000039%), followed by Pacific Islanders (0.0132%), African Americans (0.014%), Hispanics (0.027%), and Native Americans (0.11%) (44).

The H63D mutation has a broader distribution with higher frequencies throughout Europe and other geographical locations (48). The estimated prevalence of H63D homozygosity in the HEIRS study was 2.4% in non-Hispanic whites, 1.3 % in Native Americans, 1.1% in Hispanics, 0.089% in African Americans, and 0.02% in Pacific Islanders and Asians (44). The prevalence of C282Y/H63D compound heterozygosity reported in the HEIRS study was 2.0% in non-Hispanic whites, 0.77 % in Native Americans, 0.33% in Hispanics, 0.071% in African Americans, and 0.096% in Pacific Islanders, and 0.0055% in Asians (44).

SNPs in *HFE* corresponding to C282Y and H63D have been associated with iron and erythrocyte traits in healthy populations. The SNP rs1800562 that results in the C282Y variant has been associated with several iron biomarkers (59–63) and hematological parameters (64) that reflect both systemic and cellular iron homeostasis at the genome-wide level among Europeans. The SNP rs1799945 that results in the H63D variant has also been associated at the genome-wide level with various iron traits (59,65) in Europeans. In Hispanics, both rs1800562 and rs1799945 have been shown to be associated with iron status biomarkers (66). In African Americans, only the association of rs1800562 with SF and TF seen in Europeans has been

replicated (67). Noteworthy, these variants are nearly absent in Asians and Pacific Islanders (44,52), and no associations between these mutations and iron biomarkers have been reported in these populations (68).

HJV and *HAMP*

The *HJV* encodes the hemojuvelin protein which acts as a BMP co-receptor to regulate the expression of *HAMP* (21) (**Figure 4.1**). Juvenile hemochromatosis (JH) or HH type 2 is the most severe form of HH (69,70) and can arise from pathogenic mutations in *HJV* (HH subtype 2a) or *HAMP* (HH subtype 2b) and results in cardiomyopathy, diabetes and hypogonadism by the early 20s (69,70). In rare instances adult-onset HH due to *HJV* mutations has been observed (71,72). Biochemical abnormalities of JH include high TSAT and marked increases in SF (70). Interestingly, a systematic review assessing the genotypic and phenotypic spectrum of *HJV* mutations in patients with HH reported ethnic disparities in the clinical presentation of *HJV*-related HH between Europeans and East Asians (73). This same review (73), in agreement with a phenotypic analysis of HH subtypes (70) found that European males and females were affected equally, while East Asian males were affected at higher rates than their female counterparts (73).

The first comparative study of the prevalence of *HFE* and non-*HFE* related HH reported a predicted prevalence of homozygous *HJV* pathogenic mutations of 1 in 5 million using available next-generation sequence (NGS) databases, with the highest predicted prevalence in South Asians (74). Homozygous *HAMP* pathogenic mutations were predicted to be even rarer (1 in 182 million) (74). Of note, these predictions may be restricted given that the populations were not representative of all the pathogenic mutations within the HH-related genes identified to date.

Mutations in *HJV* account for up to 90% of JH cases (73), and most have been identified in a single family or small populations (73). The G320V mutation is a more common *HJV* mutation and is restricted to European ancestry (75,76). Other mutations have been described only within individuals of Asian or Pacific Island ancestry (73,77), some are more common among East Asians (e.g. C321X, Q312X) (71,78–85) and some among South Asians (e.g. G336X, G99R, P192L, L194, C80Y, A343PfsX23) (77,86,87). Few mutations have been described in Africans (e.g. R385X, A310G) (73,88,89), however, these are not restricted to African ancestry (90–93). Mutations in *HAMP* result in a rare form of JH and have been characterized in patients from varying geographical locations (87,93,102,94–101).

Interestingly, no polymorphisms within the *HJV* or *HAMP* have been associated with variation in iron traits among healthy individuals. A candidate gene association study reported an association between the SNP rs10421768 in *HAMP* and hemoglobin among a Kenyan cohort (*n* = 628) (103), but the significance of this association disappeared after adjustment for multiple testing.

TFR2

TFR2 forms a complex with HFE and possibly HJV to promote BMP-SMAD signaling and upregulate hepcidin production (29) (**Figure 4.1**). HH type 3 results from pathogenic mutations in *TFR2* and is a disease mainly of adult onset, although, more severe mutations have been described in early childhood (104–106). Biochemical abnormalities include elevated TSAT and SF (24). Although the true prevalence of *TFR2* mutations is unknown, homozygous *TFR2* pathogenic genotypes have been estimated (using available NGS databases) to be 1 in 6 million

and were predicted to be most frequent among non-Finnish European populations from ExAC database (74).

Most mutations in *TFR2* that result in HH type 3 have been clustered in ~45 families worldwide (107) but some mutations have been described among unrelated individuals from different ethnic groups (105,108–111). *TFR2*-related HH may be the leading cause of IO in Asians, predominantly those from Japan (112,113). The I238M variant is present in Asians at a higher frequency (0.0192) (112), however, it is not restricted to Asian ancestry (93).

The rs7385804 SNP in *TFR2* has been associated iron biomarkers (59,64,65) and RBC parameters (59,114) at genome-wide significance in healthy Europeans. The associations of rs7385804 with serum iron and TSAT have been replicated in Hispanics (66) and Chinese women (115). Another SNP within *TFR2*, rs7786877, has been associated with mean corpuscular volume (MCV) at the genome-wide level among individuals of European ancestry (64).

FPN

The *FPN1* gene encodes FPN, which regulates cellular export of non-heme iron (116) (**Figure 4.1**). Pathogenic mutations in *FPN1* can result in two phenotypically distinct diseases, HH type 4A (FPN disease) and HH type 4B. HH type 4B is caused by gain-of-function mutations resulting in partial or complete hepcidin resistance (117). Individuals with these mutations present with high SF, TSAT, organ IO, and progressive organ damage (117). The phenotype of HH type 4B resembles other recessive HH-causing mutations (117). Conversely, FPN disease is due to dominant loss-of-function mutations that result in impaired iron export, particularly from reticuloendothelial cells (117). The classical phenotype of FPN disease includes high SF, normal to low TSAT, low hemoglobin concentrations, and progressive iron

loading (117,118). FPN disease seems to have a milder clinical presentation as it has not shown to cause major organ damage (119,120), conceivably because macrophages protect against reactive oxygen species when burdened with iron (117). Not all FPN mutations have been classified based on their phenotypic presentation or pathogenicity (117,119).

Globally, the estimated pathogenic genotype carrier rate of *FPN1* mutations (using available NGS databases) is 1 in 1,373 (74). The highest predicted pathogenic genotype carrier rate was reported in African Americans (0.25%), followed by Americans (0.039%), East Asians (0.033%), and non-Finnish Europeans (0.03%) (74). A systematic meta-analysis of *FPN1* mutations found 31 disease-causing mutations in 161 individuals by 2010 (119), and more recently a total of 60 variants in 359 individuals was described between 1999 to 2019 (120). The most frequently reported *FPN1* mutation is the V162del loss-of-function mutation (119–126).

Few polymorphisms in *FPN1* have been associated with iron traits in healthy populations. Q248H is the most common *FPN1* variant among individuals of African ancestry and is present at polymorphic frequencies in African populations (2.2-13.4%) (127–131). The Q248H variant has been weakly associated with increased SF levels among individuals with primary IO and healthy individuals (127–129,131–133), and this association seems to be stronger in men (132,133). This polymorphism may confer a protective effect against ID (128,134), anemia, and iron deficiency anemia (IDA) (134) in African children, particularly those with underlying inflammation. While conflicting (134), a protective effect against malarial infection has also been suggested. The largest GWAS on iron status conducted in healthy Europeans identified a SNP (rs744653) near *FPN1* that was associated with SF and TF at genome-wide significance (59), and the association with SF was replicated in a recent candidate gene association study among Europeans (135). Lastly, a GWAS conducted in healthy Chinese men

found a SNP (rs5742933) located in close proximity to the *FPN1* gene that was associated with SF at the genome-wide level (136). However, this SNP seemed to be in weak linkage disequilibrium (R^2 and D'<0.20) with SNPs located within *FPN1* (136).

Genetic variants related to iron deficiency

As with IO, several SNPs in iron-related genes have been associated with increased risk of anemia, ID or IDA in healthy populations of various ethnicities. Hereditary disorders that result in anemia include iron-refractory iron deficiency anemia (IRIDA) and iron loading anemia.

TMPRSS6

MT-2 is produced in the liver and negatively regulates expression of hepcidin by cleaving HJV and in turn, inactivating the BMP-SMAD pathway (137) (**Figure 4.1**). Genetic mutations in the MT-2 protein (*TMPRSS6,* transmembrane serine protease 6) can result in uninhibited hepcidin production leading to IRIDA (138–140). This disease is characterized by congenital hypochromic, microcytic anemia, low MCV and TSAT, low to normal SF levels, and defective iron absorption and utilization (138,141). IRIDA is usually unresponsive to oral iron, and only partially responsive to parenteral iron treatment (138).

The prevalence of pathogenic mutations resulting in IRIDA is unknown (140–142). So far, 69 different *TMPRSS6* mutations in 65 IRIDA families of different ethnicities have been identified (137,141–144). Most IRIDA patients have homozygous mutations in *TMPRSS6* and thus, the mode of inheritance is considered to be recessive (143). However, heterozygous mutations may also result in a clinical phenotype that resembles IRIDA but with a milder

presentation (141,143). More recent evidence suggests that IRIDA is a highly heterogeneous disease and some patients have been found to respond to oral iron therapy (143).

Several genome-wide and candidate gene association studies conducted in healthy populations of mainly non-African origin, have found common SNPs in *TMPRSS6* to be associated with iron traits. The most reported SNP in *TMPRSS6*, rs855791 (V736A), has been associated with iron status indicators (59,62,145,146), RBC parameters (59,64,147), and liver iron content (148) in healthy individuals of European ancestry at genome-wide significance. Additionally, candidate gene studies in Europeans have replicated the association of this SNP with SF (135,149) and serum iron (149) and have identified associations with sTfR (61) and the sTfR:SF ratio (150). This SNP was also associated with hemoglobin (6,115) and iron status indicators (6,115) in East Asians, and with serum iron in Hispanics (66). A systematic review with meta-analysis found the minor allele (A) frequency (MAF) of rs855791 to be significantly higher in Asians than in Europeans (0.55 vs 0.42) (151). This same review showed that each A allele was associated with 0.11 g/dL lower hemoglobin concentrations, 3.71 µg/L lower SF levels, and 0.2 mg/L higher sTfR, and the differences in effect estimates between ethnicities were not significant (151). Significant differences in MAF between Africans and non-Africans have been reported (152). The rs855791 SNP has a significantly lower MAF in Africans (<0.1) compared to non-Africans (<0.35), yet Africans have a high prevalence of anemia (152).

The second most reported SNP in *TMPRSS6* is rs4820268 (D521D) and it has been associated with iron biomarkers (60,65,146), and hemoglobin in Europeans (147), with SF and hemoglobin in East Asians (6), and with hemoglobin in South Asians (147). The association with serum iron was replicated in a candidate gene study of Europeans and an association with SF was identified (149). A study in Europeans found the rs4820268 GG genotype to be associated with

lower serum iron, hemoglobin, MCV, and mean cell hemoglobin, and higher TF, sTfR and sTfR:SF ratio (65). Furthermore, a meta-analysis of 13 study populations showed that the G allele resulted in a 0.12 µg/L increase in SF concentrations among Europeans and a 3.69 µg/dL decrease among Asians (151). Analysis of 3 studies among Europeans suggested an association between the G allele and a reduction in sTfR (151). However, there was high heterogeneity of the effect of each allele on the variation in iron status parameters reported in studies among Europeans included in this meta-analysis (151). Consistent with these observations, a recent study assessing differences in allele frequencies among different populations found that South and East Asians had the highest number of iron lowering alleles and Africans had the lowest number of low iron risk alleles (152). Furthermore, studies in Chinese populations identified rs855791 and rs4820268 polymorphisms as genetic risk factors for developing anemia, ID, and IDA (6,115). Several other SNPs in *TMPRSS6* have been associated with RBC parameters (64,147), and iron biomarkers (146) among different ethnic groups.

TF

Autosomal recessive mutations in *TF* cause severely reduced serum concentrations of functional TF and lead to hypotransferrinemia (or atransferrinemia) **(Figure 4.1)**. This rare disorder is characterized by iron deficient erythropoiesis and anemia due to insufficient iron supply to erythropoietic tissues and severe IO in non-hematopoietic organs due to low hepcidin levels and increased non-TF mediated iron uptake (153,154). Only 18 cases among 16 families world-wide have been described to date (155,156).

While mutations that result in hypotransferrinemia are extremely rare, several SNPs in *TF* have been associated with iron status in populations from different ethnic origin. The

rs3811647 SNP in *TF* has been associated with TIBC and TF in genome-wide and candidate gene association studies in Europeans (60,63,65,150,157). Other SNPs in *TF* have been associated with serum iron, TF, and TSAT (59). Associations of several polymorphisms in *TF* with TIBC found in Europeans have been replicated in Asians, Hispanics and/or African Americans (157). Additionally, a candidate gene association study in Chinese women found *TF* polymorphisms were significantly associated with serum iron, TF, and TSAT (115). Other SNPs in *TF* have been associated with TSAT and TIBC in Hispanics (66), and with TIBC in African Americans (67). Moreover, the GWAS in African Americans found the top two SNPs in *TF* to explain 11.2% of the variation in TIBC levels in this population (67). Interestingly, unlike other iron-related genes discussed in this section, in *TF*, no SNPs have been reported to be associated with hematological traits in any ethnic group.

DMT1

DMT1 transports dietary iron into enterocytes **(Figure 4.1)** or out of intracellular endosomes. Pathogenic mutations in *DMT1* result in microcytic anemia and severe organ iron loading (141). Despite having body iron excess, patients with defects in *DMT1* have normal to mildly elevated SF levels (141). Iron loading anemia due to mutations in *DMT1* is extremely rare with only 7 patients from 6 families with homozygous or compound heterozygous described to date (141). Despite the importance of *DMT1* in iron trafficking, few polymorphisms have been associated with iron traits. In a healthy Turkish cohort, the IVS4+44 polymorphism in *DMT1* was associated with inter-individual variations in serum iron (158). This same SNP was associated with up to a four-fold increased risk of developing anemia in Italian children with Celiac disease (159).

Other genetic variants related to iron metabolism

Iron-related genes

Polymorphisms in other iron-related genes involved in transport of non-heme and heme iron (*TFR1*; *CYBRD1*, cytochrome b reductase 1; *FLVCR*, feline leukemia virus subgroup C receptor-related protein; *STEAP3*, six-transmembrane epithelial antigen of prostate 3; *CD163*, cluster of differentiation 163), in the regulation of cellular hepcidin signaling pathways (*SMAD8; BMP2; BMP4; BMP9; BMPR1B; BMPR2; NEO1*, neogenin 1; *PCSK7*, protein convertase suntilisin/kexin type 7), or intracellular iron signaling (*HIF2A*, hypoxia-inducible factor 2 alpha*; IRP1*, iron regulatory protein 1), have been associated with or have suggestive associations with at least one biomarkers of iron status and/or erythrocyte phenotype in healthy populations (59,61,135) or act as modifiers of HH phenotype (160–162) among individuals of European descent.

Non-iron related genes

Although most genetic variants associated with iron status indicators are within or in close proximity to iron-related genes, genetic variants of non-iron related genes have also been identified. Among Europeans, SNPs in genes involved in lipid metabolism were shown to be associated with TF (*NAT2*, N-acetyltransferase 2; *ARNTL*, aryl hydrocarbon receptor nuclear translocator like*; FADS2*, fatty acid desaturase 2), SF (*ABO*, alpha 1-3-N-acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase; *TEX14*, testis expressed 14) (59) or serum iron (LPL, lipoprotein lipase) (146). In Hispanics, a SNP in myelin regulatory factor (*MYRF*) that is in linkage disequilibrium with the *FADS2* SNP identified in Europeans

(59) was associated with TIBC (66). Moreover, the associations between SNPs in *NAT2* with TF and in *ABO* with SF found in Europeans (59) were generalized to Hispanics (66). Whether such associations result from the confounding influence of variations in lipid metabolism on iron status or reveal a pleiotropic effect of lipid-related genes on iron regulation needs further investigation. Interestingly, in analysis of the UK Biobank samples, ferritin-associated SNPs in *HFE* and *TMPRSS6* conferred significant protection against hypercholesterolemia, suggesting interplay of metabolic pathways between lipid and iron (163). Other SNPs in non-iron related genes have been associated with iron traits, including TIBC, unsaturated iron binding capacity, serum iron, and sTfR in Europeans (146,157).

In African Americans, SNPs in the hepatoma-derived growth factor-like protein 1 (*HDGFL1*) and MAF bZIP transcription factor (*MAF*) have been associated with TIBC and in growth factor receptor bound protein 2-associated protein 3 (*GAB3*) with SF at the genome-wide significance (67). Among these associations, only the significant SNP in *GAB3* was replicated in Hispanics (66). *GAB3* is a member of the GBR2-associated binding protein gene family and is involved in several growth factor and cytokine signaling pathways. The protein encoded by this gene is expressed in hematopoietic tissues and facilitates macrophage differentiation (164,165).

In East Asians, a SNP in postmeiotic segregation increased 1 (*PMS1*) was found to be significantly associated with SF (136). *PMS1* encodes a protein thought to be involved in DNA mismatch repair pathways and is expressed by various tissues, including hematopoietic tissues. Interestingly, anti-PMS1 antibodies were found in Japanese patients with aplastic anemia (10%) but none were detected in aplastic anemia patients from the Unites States (166). Additionally, while *PMS1* has not been shown to play a direct role in iron homeostasis, it is in close proximity to *FPN1*.

Ethnic differences in iron status

Iron regulation is highly conserved, but risk of iron related disorders differs across major ethnic groups. These ethnic differences may be a consequence of adaptive changes during evolution that occurred due to limited iron availability, a condition referred to as antagonistic pleiotropy. In antagonistic pleiotropy, adaptive changes that arise from evolutionary adaptations can become deleterious in the current environment. For iron, due to its importance in physiology, strong selective pressures would have been expected to maintain adequate iron status. These adaptations needed in an iron poor environment, *i.e.* mutations that increase iron absorption, may now be deleterious when iron is considerably more abundant in our food systems. Understanding the evolutionary context underlying dietary adaptation could have strong implications in precision nutrition (167).

Large epidemiological reports of ethnic differences in iron status indicators have been published over the past few decades. Several studies of North American cohorts have evaluated iron status in large groups of otherwise healthy adults. The largest epidemiological study to date that evaluated iron stores as a function of ethnicity was the HEIRS study. This study recruited 101,168 primary care adults aged 25 years or older from the United States and Canada, and evaluated SF and TSAT as a function of ethnicity (self-reported as Hispanic, European, African American, Asian, Pacific Islander, Native American) (168). Another large epidemiological study, the Recipient Epidemiology and Donor Evaluation Study-III recruited 12,683 men and women participants of different ethnicities (self-reported as African American, Asian, White, Hispanic, and other) aged 18 years or older that had successfully donated whole blood (169). This study was designed to examine the genetic and metabolic basis of blood donor susceptibility to ID and iron-related symptoms in multiple ethnic groups. The Iron and Atherosclerosis Study

(FeAST) was a prospective, randomized controlled single-blinded clinical trial to test whether iron reduction using phlebotomy in participants with symptomatic but stable peripheral arterial disease can effectively improve clinical outcomes. This study recruited 1,277 European and African American veterans over the age of 21 (primarily males) (170). An additional study among 1,491 African American and 31,005 European men and women compared hematologic and iron status between these two groups (171). Another study designed to determine the frequency of *HFE* mutations and its association with iron related genotypes involved 10,198 adults (self-reported as Black, Asian, White, and Hispanic) (172). Additional epidemiological data examining iron status among multiple ethnic groups was conducted using the NHANES III database involving 20,040 individuals aged 18 years or older (grouped as Black, White, Hispanic, and other) (173).

Similarly, large cohort data on iron status have been published in Europe. The population-based SUNSET study was a multiethnic, cross-sectional study designed to test the association between SF and the prevalence of type 2 diabetes and fasting glucose concentrations in a total of 2,975 adults (174). In addition, two other cohort studies targeting women of reproductive age were conducted to examine ethnic differences in iron status (175,176). Major findings from these data are summarized below by ethnicity.

Europeans

Most research to date has been conducted in Europeans, which are often used as the reference group in comparisons to other ethnic groups. Europeans have been consistently shown to have a lower risk of ID and anemia, and higher hemoglobin concentrations (171,177–180). In addition, TSAT in Europeans has been found to be significantly higher than mean values observed in African Americans and Hispanics (179,180).

African Americans

Lower hemoglobin concentrations are consistently reported among African Americans compared to Europeans. These differences have been noted in infancy and appear to be maintained in the elderly (171,176,181–184). Existing data from population groups with sample sizes ranging from 388 to 3,074 indicate that the hemoglobin distribution observed among African Americans is shifted to the left by approximately 0.8 g/dL (184,185). This difference has been highlighted by the CDC and WHO to promote ethnicity-specific cutoffs for hemoglobin in the diagnosis of anemia (37,186).

Ethnic differences in the hemoglobin distribution remain significant even after controlling for iron status (SF and TSAT) (182,184) and/or dietary iron intake (182), suggesting that variation in hemoglobin concentration is not entirely driven by factors related to iron metabolism. Despite lower hemoglobin concentrations, African American adults have elevated SF concentrations compared to Europeans and Hispanics (44,170,173,183,186–188). NHANES III (173,189), FeAST (170,188), and HEIRS (179) data all found significantly higher SF in African Americans compared to Europeans. However, both NHANES and HEIRS (179,180), and Li et al.'s genetic study in African Americans (67) found that African ancestry was associated with decreased levels of serum iron and TSAT as well as increased levels of sTfR. Moreover, prevalence of ID and anemia in HEIRS was greater in African Americans than in Europeans and Asians among women of reproductive age (177,190,191). At present, whether the elevated SF concentrations in African Americans reflects increased systemic inflammation or elevated iron stores is unclear. African American populations are often at increased risk for obesity compared to other ethnic groups (192), and elevated BMI is positively correlated with SF concentrations, perhaps due to adiposity-induced inflammation. The lower TSAT and

hemoglobin concentrations but higher SF levels observed in African Americans might indicate that the mobilization of iron from stores for erythropoiesis is reduced due to some unknown genetic contributors (182).

East Asians

Asian populations can be further sub-divided into East Asians or South Asians based on geographical distribution and ethnicity. Although data in Asian groups are not as abundant, numerous studies have reported that East Asian populations exhibited higher iron stores and a higher risk of IO. This finding is evident despite the fact that the frequencies of the most common *HFE* mutations are lowest among Asians (44). Of note, HEIRS individuals that self-identified as Asians (predominantly East Asians: Chinese, Japanese, Vietnamese, and Filipino) exhibited the highest SF, TSAT or both SF and TSAT compared to any other population group studied, even after excluding polymorphisms associated with IO (44,191,193). These differences remained significant after adjusting for diabetes or liver disease (193). In a further analysis of HEIRS data focused on women of reproductive age, Asian ancestry was associated with a decreased risk of ID and with increased iron stores compared to other ethnic groups independent of known HH mutation in *HFE* (C282Y) (191). Additional epidemiologic data from a study in Korean adults (n = 4,904) found mean TSAT in both females and males was significantly higher than in Europeans as reported in the HEIRS study (194,195). Few data exist on possible mechanisms explaining these ethnic differences. A recent functional study evaluated iron absorption in a group of young East Asian women (32) and found mean percent iron absorption was significantly higher in East Asian women compared to that reported in European women using the same methodology, even after correcting for a fixed amount of SF (32). This observation suggests that the storage threshold at which East Asians down-regulate iron

absorption is higher, supportive of increased risk of IO in East Asian populations at maturity. This may also explain the observation that East Asian populations have a greater risk of diabetes at a lower BMI (196,197).

South Asians

Fewer data are available from South Asian populations, but existing data in this group suggests a lower iron status compared to Europeans and East Asians. Data from South Asian Surinamese adults (Hindustani Surinamese, n = 399) aged 35-60 years reported a significantly lower SF concentrations than observed among a cohort of Dutch adults (n = 508) (174). South Asian pregnant women (primarily from Pakistan and Sri Lanka, n = 198) were reported to have the highest risk of ID and anemia when compared to pregnant European (n = 326) and East Asian women (n = 43) (177). Lower hemoglobin, SF, and TSAT have also been noted in a group of South Asian women of reproductive age (176).

Hispanics

At least two epidemiological studies have reported a significantly higher prevalence of ID in Hispanics (178,190) compared to other ethnic groups, and Hispanic ethnicity has been associated with an increased risk of ID (191). In addition, evidence from NHANES found that Hispanic women of reproductive age had significantly lower SF and TSAT compared to the European women (180).

Native Americans

Data on iron status among Native American populations are limited. The HEIRS study included data from 645 Native Americans. In this relatively small cohort, mean TSAT and SF

concentrations in Native American men and women did not appreciably differ from the respective mean values reported in European men and women (198).

Statistical evaluation of population-based data on iron status

Multiple published reports of ethnicity and iron status exist but few attempts have been made to compile these data to explore statistical patterns of altered iron status. To address this gap, relevant population-based data were identified through PubMed, Web of Science, and Scopus using the following key words: ethnicity, race, genetic, Asian, European, Caucasian, African American, Chinese, Korean, genetics, iron, iron status, iron homeostasis, iron absorption, iron metabolism. Additional studies were identified through references cited within relevant articles. To systematically compare the data summarized above on iron status among different ethnical groups, we extracted iron status data from both population-based studies involving multiple ethnicities and other epidemiological or observational studies reporting data from one ethnic group only. We categorized studies by the number of ethnic groups involved and by the study type (**Appendix Table 3**). For analytical purposes, we excluded studies designed to assess iron status in frequent blood donors or pregnant and/or breastfeeding women, as this would be expected to impact iron status. Studies were also excluded if there were no available iron status data to extract or if the iron status data were not reported by sex. As for different literature examining the same study cohort, data from the largest sample size were included in the analyses.

To explore possible differences in iron status indicators (SF, TSAT and hemoglobin) between different ethnic groups, linear mixed-effects models were constructed with ethnicity (East Asian, European, African American and South Asian) as a fixed-effect variable. Studies

where the data were collected from were considered as a random-effect variable. Sample size of each ethnic group in these studies was used as weight for the fixed-effect variable and the mean age of each ethnic group was controlled in the analysis. Mean age could not be controlled for models examining hemoglobin as this restricted the size of the dataset because many of the available studies did not report the mean age. Estimated marginal means of each iron status indicator were calculated for all ethnic groups using the package *emmeans* in R. All statistical analyses were performed using R version 3.4.3.

The estimated marginal means of iron status indicators evaluated are presented in the **Table 4.1**. A statistical evaluation of mean differences in each indicator between ethnic groups is presented in **Table 4.2**. With this approach, Asians (predominantly East Asians) were found to exhibit significantly higher SF concentrations and TSAT when compared to Europeans, African Americans or South Asians, and this difference was significant for both males and females. While significant effects were evident in both sexes, the magnitude of the observed difference was significantly higher in males. This finding is expected as women of reproductive age have monthly losses of iron from menses, which may partially explain why risk of excess iron accumulation is greater in males. Evidence has shown that 1 µg/L of SF is equivalent to 8 mg of iron stores (199), thus the mean difference of 180 µg/L in SF would be translated into an additional 1440 mg of storage iron. If 1.5 mg of iron is absorbed daily (21), this additional 1440 mg of storage iron would be equivalent to the net amount of iron typically absorbed over 2.6 years.

Hemoglobin concentrations are impacted only when iron stores have been depleted (200). African American men were found to have significantly lower hemoglobin compared to European, Asian or Hispanic men. Similarly, African American women had significantly lower

hemoglobin when compared to European women. These findings are consistent with previous literature evaluating hemoglobin as a function of ethnicity (171,176,181–185).

Table 4.1 Mean reported iron status indicators in different ethnic groups[1]

	Men			Women		
	SF, *μg/L*	**TSAT, *%***	**Hb, *g/dL***	**SF, *μg/L***	**TSAT, *%***	**Hb, *g/dL***
Asian	249 ± 17 [14] (40,874)	37 ± 1 [6] (22,543)	15.1 ± 0.1 [8] (20,265)	113 ± 7 [13] (31,036)	29 ± 1 [7] (13,075)	13.1 ± 0.1 [9] (9,706)
European	68 ± 16 [15] (48,115)	32 ± 1 [8] (38,532)	14.8 ± 0.1 [10] (28,169)	27 ± 7 [20] (62,736)	25 ± 1 [10] (45,433)	13.5 ± 0.1 [13] (33,352)
African American	87 ± 16 [9] (14,025)	30 ± 1 [8] (13,839)	14.2 ± 0.1 [5] (2,882)	45 ± 7 [8] (20,413)	22 ± 1 [7] (19,908)	12.7 ± 0.2 [4] (1,350)
South Asian	57 ± 35 [1] (150)	32 ± 5 [1] (150)	-	35 ± 16 [2] (488)	18 ± 2 [2] (242)	12.6 ± 0.5 [1] (188)
Hispanic	151 ± 83 [2] (5,549)	29 ± 4 [1] (5,122)	15.0 ± 0.2 [1] (427)	54 ± 35 [2] (7,784)	25 ± 4 [1] (7,241)	13.3 ± 0.3 [1] (543)

[1] Data are presented as estimated marginal means ± SE [number of studies included in the analyses] (number of people included). The estimated marginal means were calculated from linear mixed-effects models using the *lmer* and *emmeans* functions. Hb, hemoglobin; SF, serum ferritin; TSAT, transferrin saturation.

Table 4.2 Comparison of iron status indicators between comparison ethnic groups and reference ethnic groups[1]

Iron indicator	Reference ethnic group	Comparison ethnic groups	Men		Women	
			Differences ± SE	N[2]	Differences ± SE	N[2]
SF, *µg/L*	Asian			[14] (40,874)		[13] (31,036)
		European	-180 ± 5[3]	[15] (48,115)	-86 ± 3[3]	[20] (62,736)
		African American	-161 ± 7[3]	[9] (14,025)	-68± 3[3]	[8] (20,413)
		South Asian	-192 ± 30[3]	[1] (150)	-78 ± 16[3]	[2] (488)
	European			[15] (48,115)		[20] (62,736)
		African American	19 ± 4[4]	[9] (14,025)	19 ± 2[3]	[8] (20,413)
TSAT, *%*	Asian			[6] (22,543)		[7] (13,075)
		European	-5 ± 1[3]	[8] (38,532)	-3 ± 0[3]	[10] (45,433)
		African American	-7 ± 1[3]	[8] (13,839)	-6 ± 0[3]	[7] (19,908)
		South Asian	-5 ± 5	[1] (150)	-11 ± 2[3]	[2] (242)
	European			[8] (38,532)		[10] (45,433)
		South Asian	-1 ± 5	[1] (150)	-7 ± 2[5]	[2] (242)
		African American	-2 ± 1[6]	[8] (13,839)	-3 ± 0[3]	[7] (19,908)
Hb, *g/dL*	African American			[5] (2,882)		[4] (1350)
		Asian	0.8 ± 0.1[3]	[8] (20,265)	0.4 ± 0.2	[9] (9,760)
		European	0.6 ± 0.1[3]	[10] (28,169)	0.8 ± 0.2[3]	[13] (33,352)
		Hispanic	0.8 ± 0.1[3]	[1] (427)	0.6 ± 0.3	[1] (543)
	Asian			[8] (20,265)		[9] (9,760)
		European	-0.2 ± 0.1	[10] (28,169)	0.4 ± 0.1[7]	[13] (33,352)

[1]The estimated differences were calculated from linear mixed-effects models using the *lmer* and *emmeans* functions. *P*-values reported were correction for multiple comparisons with Tukey's test. Hb, hemoglobin; SF, serum ferritin; TSAT, transferrin saturation.

[2]N, [number of studies included in the analyses] (number of people included)

[3]Significant difference between the comparison group and the reference group, $P < 0.0001$.

[4]Significant difference between the comparison group and the reference group, $P = 0.0002$.

[5]Significant difference between the comparison group and the reference group, $P = 0.005$.

[6]Difference between the comparison group and the reference group approached significance, $P = 0.05$.

[7]Significant difference between the comparison group and the reference group, $P = 0.03$.

Conclusions

This review highlighted the shared and unique genetic variants among ethnic groups that have been associated with iron status biomarkers, and possible differences in iron status as a function of ethnicity were explored using published data. To date, most genome-wide and candidate gene association studies on iron homeostasis have been conducted in European populations. Some of the associations found in Europeans have been replicated in other ethnic groups but the clinical significance is unknown given the varying minor or effect allele frequencies among different populations. Interestingly, reported frequencies of key genetic variants associated with iron traits among different ethnic groups do not fully reflect epidemiological data on iron status in different populations. Because iron traits may be influenced by a combination of genetic, dietary, and lifestyle factors, methods taking into account several variants together, such as polygenic risk scores, may be better at predicting the risk of ID or IO in specific populations. Moreover, although recent studies have identified variants in non-iron related genes that are associated with iron traits, how they vary as a function of ethnicity and the significance of these SNPs has yet to be determined. Finally, most genome-wide and candidate gene association studies have been focused on blood biomarkers of iron status. Although iron status biomarkers correlate with body iron stores, their levels are sensitive to diet and disease, which may have confounded current association studies. Iron homeostasis in humans depends predominantly on the tight regulation of dietary iron absorption by enterocytes. Therefore, attempts to characterize genetic determinants of interindividual variation in iron homeostasis using direct measures of nutrient utilization are needed in order to better understand the differences in iron homeostasis that exist among different populations. Additional data are needed to identify possible risks and benefits associated with universal iron supplementation

policies such as those currently recommended for pregnant North American women (201), to identify the genetic basis of population differences in iron metabolism and disease susceptibility, and to help inform population-specific dietary iron intake recommendations and surveillance in at-risk populations.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

Summary

This doctoral research investigated three major factors that influence iron homeostasis, namely physiological, environmental, and genetic factors. Each of these factors was a specific aim of the dissertation. To address a physiological determinant of iron homeostasis, this research provided a large normative analysis of determinants of maternal Hb and identified factors associated with risk of maternal anemia and elevated maternal Hb concentrations in healthy U.S. pregnant women. To address an environmental situation that impacts iron homeostasis, the impact of parasitic infections and iron supplementation on iron absorption was investigated and identified variables associated with the change in RBC iron isotope enrichment over a 3-month period was evaluated among a group of toddlers residing in a malaria and helminth endemic environment. As for the genetic control of iron homeostasis, this doctoral dissertation summarized published literature on genetic determinants of iron status, characterized iron status in different ethnic populations, and statistically evaluated differences in iron status as a function of ethnicity using published data. These three specific aims were presented in Chapters Two through Four. Below is a brief summary of the conclusions from each specific aim, followed by implications and suggestions for future research.

The data from the specific aim one provided one of the largest normative longitudinal analyses of Hb concentrations across pregnancy in otherwise healthy U.S. pregnant women receiving routine prenatal care. Findings from Aim 1 indicated that anemia was prevalent in otherwise healthy U.S. pregnant women receiving standard prenatal care even with the current recommendation of universal iron supplementation of U.S. pregnant women. The prevalence of anemia increased as pregnancy progressed and by late pregnancy one-quarter of women evaluated had anemia. We reported that the current CDC anemia cutoffs (that were based on the

5th percentile of a 1970's reference population) may overestimate the prevalence of anemia in late pregnancy based on our more recent analysis of a much larger and more racially diverse population. Risk factors for anemia were identified as Black race or Hispanic ethnicity, being underweight at entry into pregnancy, being multiparous or carrying a multiple birth pregnancy. Identifying these risk factors may help inform targeted iron supplementation recommendations in obstetric clinical practice. These data have contributed to the current knowledge of hemoglobin concentrations in U.S. pregnant women and identified additional risk factors associated with increased risk of anemia across pregnancy.

The main findings from the Aim 2 provided a better understanding of the impact of parasitic infections and iron supplementation on iron metabolism in toddlers residing in a malaria and helminth endemic environment. This study was one of the largest stable iron isotope studies and it uniquely followed the change in RBC iron isotopic enrichment over an 84-day period post-dosing in otherwise healthy toddlers residing in a malaria- and helminth-endemic area. The strongest determinant of RBC iron incorporation was TBI. TBI likely better captures iron demands as it is calculated using biomarkers of tissue demand and storage iron. We also reported that toddlers who received iron supplementation exhibited significantly greater loss of RBC iron isotope enrichment over time. This greater loss of RBC iron enrichment may be indicative of increase in size of RBC pool and increased erythropoietic demands due to the provision of iron among anemic or helminth infected toddlers.

The findings from Aim 3 highlighted the shared and unique genetic variants that have been associated with iron status biomarkers and explored possible differences in iron status as a function of ethnicity using published data. For the first time, we pooled existing published data and demonstrated that East Asians had significantly higher iron status (serum ferritin, transferrin

saturation) compared to Europeans, African Americans, or South Asians. Supportive of other published data we also found that Black adults exhibited significantly lower Hb concentrations compared to other ethnic groups. These data contribute to this literature and may help inform subsequent population-specific recommendations and personalized nutrition interventions for iron-related disorders.

Future Directions

This doctoral research provided novel insights into physiological, environmental and genetic factors that impact iron homeostasis. These data also lead to new research questions. Further studies are needed to evaluate the impact of maternal anemia on iron status in the newborn at birth and to characterize its potential impact on birth outcomes. Targeted identification of women at greater risk for ID and IDA may help inform interventions to improve the in-utero environment in support of healthy birth outcomes. Additionally, for environmental factors, future longer-term stable iron isotope studies may provide greater insight into whole body iron homeostasis and adaptations to parasitic infections or iron supplementation in vulnerable age groups such as children. Lastly, more genome-wide and candidate gene association studies of iron homeostasis are needed in ethnically diverse populations. Additional characterization of the genetic basis of ethnic differences in iron metabolism and on how this affects disease susceptibility among different ethnic groups are needed to help inform population-specific dietary iron intake recommendations and to help screen at-risk populations. Multidisciplinary approaches that consider physiological, environmental and genetic factors may best predict iron disorders in specific populations and help develop the most effective interventions to maintain adequate iron status.

APPENDIX

Appendix Table 1. Commonly utilized biochemical indicators of iron status[1]

Biomarker	Function of Indicator	Confounders	Cut-offs in Non-pregnant Healthy Adults
SF	Indicator of storage iron	Inflammation and infection; menstruation and childbirth; blood donations; alcohol intake; liver disease and malignancy	**Deficiency:** <12-15 μg/L(1) **Overload:** > 150 μg/L in menstruating females > 200 μg/L in males and non-menstruating females (1)
sTfR	Indicator of tissue iron availability	Rate of erythropoiesis; chronic disease; sTfR assay dependent (Ramco/other ELISA kits or Roche)	**Deficiency:** >8.5 mg/L if utilizing Ramco assay (2), or >4.4 mg/L for women and >5.0 mg/L for men if utilizing the Roche Hitachi analyzer (3) **Overload:** No set cut-off
ZPP or EP	Indicator of iron supply to bone marrow	Rate of erythropoiesis; lead poisoning; low diagnostic sensitivity and specificity; high day-to-day variation	**Deficiency:** >40-80 μg EP/dL of red cells (4) **Overload:** No set cut-off
SI	Circulating transferrin-bound iron	Infection and inflammation; varies from day-to-day and after meals; under circadian rhythmicity; sample easily contaminated	**Deficiency:** <50-60 μg/dL (4) **Overload:** No set cut-off
TIBC	Measure of the total number of transferrin binding sites per unit volume of plasma or serum	Large overlap between normal values and values in iron deficiency	No set cut-offs. TIBC by itself is not used as a measure of iron status
TSAT	Indicator of iron supply to tissues (Formula = serum iron/TIBC)	Same as serum iron and TIBC	**Deficiency:** <15% (4) **Overload:** >45% (4)

Appendix Table 1 (Continued)

Hb	Measure of anemia and proxy of functional iron	Low sensitivity/specificity; does not detect recent changes	**Deficiency:** 12 g/dL in women 13 g/dL in men (5) **Overload:** No set cut-off
Soluble transferrin receptor to serum ferritin ratio	Represents iron status in the setting of inflammation, infection or chronic disease (Formula = sTfR/SF)	sTfR is assay dependent	**Deficiency:** No set cut-off **Overload:** No set cut-off
TBI	Provides a measure of the magnitude of iron deficiency that is independent of the hemoglobin concentration. Helps distinguish between iron deficiency anemia and anemia of chronic disease (Formula = - [log (serum transferrin receptor/serum ferritin) – 2.8229]/0.1207)	Same as sTfR and SF	**Deficiency:** <0 mg/kg body weight (6) **Overload:** No set cut-off

[1]EP, erythrocyte protoporphyrin; Hb, hemoglobin; MCH, mean cell hemoglobin; MCV, mean cell volume; SF, serum ferritin; SI, serum iron; TBI; total body iron; TF, transferrin; TIBC, total iron binding capacity; TSAT, transferrin saturation; UIBC, unsaturated iron binding capacity; ZPP, zinc protoporphyrin.

Appendix Table 2. Genetic variants shown to be associated with biomarkers of iron status in healthy populations stratified by ethnic group[1,2]

Chromosome	Gene(s)[3]	Variants[4] (amino acid change)	Iron Status-Related Phenotype	References
Europeans				
6	*HFE*[5]	rs1800562 (C282Y), rs1799945 (H63D)	SF, SI, TF, TIBC, TSAT, SF, sTfR, sTfR:SF, UIBC	(7,8,17,9–16)
3	*TF*[5]	rs8177240, rs8177179, rs1799852 (L247L), rs3811647, rs1358024, rs452586, rs4428180, rs8177224, rs3811658, rs8177248, rs1880669, rs1525892, rs9824452, rs7638018, rs2280673	SI, TF, TSAT, TIBC, UIBC	(7–9,11–13, 15,16)
22	*TMPRSS6*[5]	rs855791 (V736A), rs4820268 (D521D), rs228918, rs228919, rs228921, rs5756520, rs2111833, rs2235324, rs1421312, rs2743825	sTfR, sTfR:SF, SI, TF, TSAT, SF	(7–15,18)
2	*FPN1*[5]*/ WDR75*	rs744653	TF, SF	(9,10)
3	*TFR1 (TFRC)*[5]	rs9990333	TF	(9)
7	*TFR2*[5]	rs7385804	SI, TSAT	(8,9)
6	*SLC17A1*	rs17342717	SF	(8)
11	*PCSK7*	rs236918	*SF*	(10,14)
9	*ABO*	rs651007	SF	(9)
17	*TEX14*	rs411988	SF	(9)
11	*ARNTL*	rs6486121	TF	(9)
11	*FADS1*	rs174577	TF	(9)
8	*NAT2*	rs4921915	TF	(9)
3	*SRPRB*	rs9843728, rs1830084, rs6794676, rs6794945, rs13061203, rs9853615	TIBC, UIBC	(12)
18	*KIAA1468*	rs9948708, rs11877669	TIBC, SI	(12,18)
20	*TMEM90B*	rs11700002	TIBC	(12)
7	*RPA3*	rs10263415	sTfR	(12)
8	*SAMD12*	rs2460970, rs880034	TBI; SF	(12)
1	*SLC44A3*	rs735937	SI	(18)

Appendix Table 2 (Continued)

2	*CAPN13*	rs17010141	SI	(18)
3	*GRM7*	rs11131063, rs1353828, rs9880404, rs965170, rs10866078	SI	(18)
5	*TGFBI*	rs756462	SI	(18)
6	*CD109*	rs9442947	SI	(18)
7	*MAD1L1*	rs1176717	SI	(18)
8	*LPL*	rs270	SI	(18)
9	*STOM*	rs16910559	SI	(18)
11	*NELL1*	rs1670646	SI	(18)
14	*TTC7B*	rs1286474	SI	(18)
15	*SHC4*	rs16961946	SI	(18)
18	*DLGAP1*	rs17725070	SI	(18)
18	*PTPRM*	rs7505939	SI	(18)
Asians				
22	*TMPRSS6*[5]	rs855791 (V736A), rs4820268 (D521D), rs2413450	SI, TSAT, FEP, SF	(19,20)
3	*TF*[5]	rs3811647	TF, TIBC	(19)
7	*TFR2*[5]	rs7385804	SI	(19)
2	*PMS1/ FPN1*[5]	rs574933, rs3791770, rs3791773	SF	(21)
2	*ANKAR*	rs1550388, rs1225101	SF	(21)
2	*OSGEPL1*	rs1898569, rs4666783	SF	(21)
2	*ORMDL1*	rs2289404	SF	(21)
Africans				
3	*TF*[5]	rs8177253	TIBC	(22)
X	*G6PD/ GAB3*	rs141555380	SF	(22)
6	*HDGFL1/ NRSN1*	rs115923437	TIBC	(22)
16	*MAF/ DYNLRB2*	rs16951289	TIBC	(22)
10	*CUBN*	rs10904850	SI	(12)
Hispanics				
6	*HFE*[5]	rs1800562 (C282Y), rs1799945 (H63D)	TIBC, TSAT, SF, SI	(23)
3	*TF*[5]	rs4637289, rs2692666, rs1405023, rs6762719	TIBC, TSAT	(23)
22	*TMPRSS6*[5]	rs855791 (V736A)	TSAT, SI	(23)
8	*PPP1R3B*	rs4841132	TIBC	(23)

Appendix Table 2 (Continued)

11	*FADS2/ MYRF*	rs174529	TIBC	(23)
X	*GAB3*	rs141555380	SF	(23)

[1]*ABO*, alpha 1-3-N-acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase; *ANKAR*, ankyrin and armadillo repeat containing ; *ARNTL*, aryl hydrocarbon receptor nuclear translocator like; *CAPN13*, calpain 13; *CD109*, cluster of differentiation 109; *CUBN*, cubilin; *DLGAP1*, DLG associated protein 4; *DYNLRB2*, dynein light chain roadblock-type 2; *FADS1*, fatty acid desaturase 2; *FPN1*, ferroportin 1; *G6P*, glucose-6-phosphate dehydrogenase; *GAB3*, growth factor receptor bound protein 2-associated protein 3; *GRM7*, glutamate metabotropic receptor 7; *HDGFL1*, hepatoma-derived growth factor-like protein 1; *HFE*, high iron protein; *KIAA1468*, RAB11 binding and LisH domain, coiled-coil and HEAT repeat containing; *LPL*, lipoprotein lipase; *MAD*, MAX dimerization protein 1; *MAD1L1*, mitotic arrest deficient 1 like 1; *NAT2*, N-acetyltransferase 2; *NELL1*, neural EGFL like 1; NRSN1, neurensin 1; *ORMDL1*, ORMDL sphingolipid biosynthesis regulator 1; *OSGEPL1*, O-sialoglycoprotein endopeptidase like 1; *PCSK7*, protein convertase suntilisin/kexin type 7; *PMS1*, postmeiotic segregation increased 1; *PPP1R3B*, protein phosphatase 1 regulatory subunit 3B; *PTPRM*, protein tyrosine phosphatase receptor type M; *RPA3*, replication protein A3; *SAMD12*, sterile alpha motif domain containing 12; SF, serum ferritin; *SHC4*, SHC adaptor protein 4; SI, serum iron; *SLC44A3*, solute carrier family 44 member 3; *STOM*, stomatin; *SLC17A1*, solute carrier family 17 member 1; *SRPRB*, signal recognition particle receptor subunit beta; TBI, total body iron; *TEX14*, testis expressed 14; *TF*, transferrin; TF, transferrin; *TFR1*, transferrin receptor 1; *TFR2*, transferrin receptor 2; *TGFB1*, transforming growth factor beta 1; TIBC, total iron binding capacity; *TMEM90B*, synapse differentiation inducing 1; *TMPRSS6*, transmembrane serine protease 6; TSAT, transferrin saturation;*TTC7B*, tetratricopeptide repeat domain 7B; UIBC, unsaturated iron binding capacity; *WDR75*, WD repeat domain 75.

[2]Both genome-wide and candidate gene association studies included. All studies adjusted for varying covariates in their analyzes (*i.e.* sex, age, principal component scores, among other study-specific variables).

[3] Variants within or in close proximity to specified gene(s).

[4] Variants shown to be associated with variation in one or more of the iron status indicators listed on the iron status-related phenotypes column. Only associations that remained significant after multiple testing correction are included. Only SNPs with known genes nearby included.

[5] Known iron-related genes.

Appendix Table 3. Summary of literature on iron status in different ethnic populations[1]

	Ethnic groups comparison	Literature with characterization data	Literature with clinical correlates data
Studies designed to evaluate ethnicities differences in iron status	European vs. African American vs. Asian vs. Hispanic	(24),(12,25–27)[2],(28)[3]	(29)[2],(30)[4]
	European vs. African American vs. Hispanic	(31)[2],(32)[3]	-
	European vs. African American vs. Asian	(33)	(34)
	European vs. African American	(35–39), (40,41)[2],(42,43)[5]	(44)[2]
	European vs. Asian	(45),(46)[5]	-
	European vs. Native American	(47)	-
Studies characterizing iron status in single ethnicity	European	(48,49,58–62, 50–57),(63)[3]	-
	Asian	(64–67),(21,68–71)[2]	(20,72–81), (82,83)[2],(84)[6]
	African American	(22)	-
	Hispanic	-	(23)

[1]The study type was categorized as either a study characterizing iron status in large cohorts or a study designed to compare case-control to a specific clinical condition. The most common clinical conditions that might be associated with iron status examined in the literature are iron deficiency, anemia, iron overload, diabetes, and metabolic syndrome.

[2]Literature was excluded from statistical analyses because of repeat study cohort.

[3]Literature was excluded from statistical analyses because pregnant and/or breastfeeding women were the study subjects.

[4]Literature was excluded from statistical analyses because frequent blood donors were the study subjects.

[5]Literature was excluded from statistical analyses because iron status data were not available.

[6]Literature was excluded from statistical analyses because there was no iron status reported by sex.

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