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EFFECT OF TOTAL BODY IRON ON METABOLIC DYSFUNCTION AMONG U.S. FEMALES IN THE NHANES 2003-2010 SURVEY, AGED 12-49

By

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MA, University of Louisville, 2010
BS, Morehead State University, 2000

A Dissertation
Submitted to the Faculty of the
School of Public Health and Information Sciences of the University of Louisville
in Partial Fulfillment
of the Requirements for the Degree of

Doctor of Philosophy in
Public Health Science

Department of Epidemiology and Population Health
University of Louisville
Louisville, Kentucky

December 2017

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December 05, 2017

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DEDICATION

This Dissertation is dedication to my parents

Mrs. Maguana Carhart-Qualls

and

Mr. Michael Qualls

who have provided me with invaluable support and educational opportunities.

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I would like to thank my advisor and dissertation director, Dr. Frank Groves, for his guidance, patience and humor during our research. I would also like to thank the other committee members for their guidance and commentary over the past 6 years. Moreover, I would like to express my thanks and gratitude to my wife, Andrea, for her patience and understanding during those times when I would say “Just a little bit longer. I can see the light at the end of the tunnel” and yet it seemed quite often that no end was in sight. This entire process has thus been a team-effort and I am happy to say that we have finally made it.

ABSTRACT

EFFECT OF TOTAL BODY IRON ON METABOLIC DYSFUNCTION AMONG U.S. FEMALES IN THE NHANES 2003-2010 SURVEY, AGED 12-49

Joseph M. Carhart

December 05, 2017

Experimental and epidemiological studies have reported positive associations between iron parameters and metabolic dysfunction including: Type 2 diabetes mellitus (T2DM) (1-6), metabolic syndrome (7-15), non-alcoholic fatty liver disease (NAFLD) (16-22) and insulin resistance (23,24) with a number of studies showing that reductions in total body iron via dietary modification (e.g., reducing red meat intake) or phlebotomy lead to increases in insulin sensitivity (25), decreases in insulin resistance (26), and reductions in the prevalence of complications associated with T2DM (5, 27), metabolic syndrome (28) and NAFLD (22, 29). Moreover, the risk associated with these outcomes and total body iron differ between males and females and it has been hypothesized that the divergence in risk of disease due to total body iron is related to the female reproductive lifespan (e.g., age at menarche, parity, oral contraceptive use and age at menopause). Regardless of study design, serum ferritin and transferrin saturation have consistently been used in these investigations to estimate total iron stores, which are affected by inflammation and demonstrate diurnal variation.

The aim of the current study was to examine the clinical utility of an index of iron repletion developed by JD Cook et al. in 1993 (i.e. Fe_{COOK}), which is unaltered by inflammation or liver function in a nationally-representative, community-dwelling, non-institutionalized group of females participating in continuous NHANES from 2003 to 2010 and to examine the association between the index and metabolic dysfunction.

The current results highlight the clinical utility of Fe_{COOK} in assessing the iron status in females of reproductive age. I did not observe a statistically significant association between metabolic dysfunction and continuously-scaled Fe_{COOK}. However, when Fe_{COOK} was modeled in accordance with common reporting patterns (i.e., categorically), significant, positive associations between increasing Fe_{COOK} and metabolic dysfunction were observed among all females, and this pattern persisted after stratification by ethnicity and menopausal status. Further research is warranted to examine the clinical utility of Fe_{COOK} among other cohorts. Moreover, additional research using Fe_{COOK} is recommended in order to replicate these findings among a larger sample comprised of both males and older females.

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CHAPTER 1: INTRODUCTION

Experimental and epidemiological studies report positive associations between iron parameters and metabolic dysfunction including: Type 2 diabetes mellitus (T2DM) (1-6), metabolic syndrome (7-15), non-alcoholic fatty liver disease (NAFLD) (16-22) and insulin resistance (23, 24). While much consensus in the research community has been reached regarding the ascertainment of T2DM, metabolic syndrome and insulin resistance in population-based studies using World Health Organization (WHO) criteria, National Cholesterol Education Program-Adult Treatment Panel III (ATP III) criteria and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), respectively, no gold-standard method exists for the detection of hepatic steatosis that is both non-invasive and cost-effective. Therefore, several algorithms which utilize markers found in serum have been developed to estimate the presence of fatty liver in large cohorts (Table 7). Among these algorithms, the fatty liver index (FLI) has received the most attention in the epidemiological literature (30) and is the only algorithm that has been externally validated in four independent studies (31-34, Table 7) since its inception in 2006 (30). More recently, an index referred to as the U.S.-FLI has been developed to determine the presence of NAFLD among multi-ethnic cohorts. Unlike the FLI, the U.S.-FLI accounts for age and ethnicity and has been shown to outperform its predecessor (i.e., the FLI) (35) However, the U.S.-FLI has not been employed thus far to estimate the prevalence of NAFLD in U.S. females as a subgroup.

The magnitude of risk in the above reported associations between iron stores and metabolic dysfunction differ between males and females and it is thought that the divergence in risk of disease due to body iron status is related to the female reproductive lifespan. In his 1981 article entitled “Iron and the sex difference in heart disease risk” Jerome Sullivan was the first to hypothesize that blood loss during menstruation is the primary factor that protects premenopausal females from ischemic heart disease (36). In the years that followed, several publications have reported that parameters related to ovulation and menstruation (e.g., decreasing age at menarche, increasing parity, decreasing length of oral contraceptive use and increasing age at menopause) confer protection against chronic disease (37-40) and that this protection appears to be lost following the onset of menopause (38). Supporting these observations are clinical trials, cohort studies and case-control studies whose results consistently demonstrate that participants who reduce total body iron via dietary modification (e.g., reducing red meat intake) or blood donation/phlebotomy experience increases in insulin sensitivity (25), decreases in insulin resistance (26), and reductions in the prevalence of complications associated with T2DM (27), metabolic syndrome (42) and NAFLD (43). Regardless of study design, however, serum ferritin and transferrin saturation have consistently been used in these investigations to estimate iron stores. Despite its clinical utility, ferritin is adversely affected by inflammation, with levels of serum ferritin rising under inflammatory conditions (44). In addition, transferrin saturation (TSAT), which is the ratio of serum iron to total iron binding capacity (TIBC), demonstrates diurnal variation (45) and increases in the presence of health conditions such as inflammation, cirrhosis, hepatitis and microbial infection (46-49).

Predictive, non-invasive, and inexpensive analytes unaltered by inflammation or liver function would be of value in monitoring the iron status of females across the reproductive lifespan to distinguish the effects of iron stores on metabolic dysfunction. Moreover, reproductive parameters vary between females and this offers a natural setting in which to examine the effect of these parameters on iron stores and their association with metabolic dysfunction while controlling for the effect of covariates such as age, ethnicity, education, body mass index (BMI) and lifestyle factors (e.g., diet composition (i.e., foodstuffs and supplements which enhance and/or inhibit iron absorption), alcohol intake and smoking status (i.e., exposures which increase iron loading) and physical activity (i.e., an exposure which decreases iron loading).

In this secondary analysis, my aim was to test the effect of reproductive parameters on iron stores and to evaluate body iron as a predictor of metabolic dysfunction in female participants of childbearing age (i.e., 12-49 years) participating in the continuous National Health and Nutrition Examination Survey (NHANES) from 2003-2010. To estimate iron stores in the current sample, I employed a well-validated algorithm developed by JD Cook et al. in 2003 (50) which is a mathematically-derived ratio of soluble transferrin receptor and ferritin found in serum and appears to be a more stable index of iron stores than either analyte used independently (51-52).

Aim 1: To estimate iron stores in a nationally-representative, community-dwelling, non-institutionalized group of females participating in continuous NHANES from 2003 to 2010 using an index of iron repletion developed by JD Cook et al. in 2003.

Aim 2: To describe the relationships between age, ethnicity, education, BMI, alcohol consumption, smoking history, physical activity, dietary iron, foodstuffs which enhance/inhibit dietary iron absorption, reproductive parameters, and Fe_{COOK} in the female cohort. Specifically, it was hypothesized that:

A. Fe_{COOK} would be positively associated with

1. Hispanic and Non-Hispanic Caucasian ethnicity
2. Education
3. BMI
4. Alcohol consumption
5. Smoking history
6. Dietary iron intake
7. Length of oral contraceptive use
8. Decreasing age at menopause

B. Fe_{COOK} would be negatively associated with

1. Non-Hispanic African American ethnicity
2. Physical activity
3. Decreasing age at menarche
4. Parity

Aim 3: To estimate the prevalence of non-alcoholic fatty liver disease in the current sample using a validated algorithm developed by Ruhl et al. in 2015 (35), the U.S. fatty liver index (U.S.-FLI).

Aim 4: To describe the relationship between Fe_{COOK} and risk of metabolic dysfunction in the female cohort. It is hypothesized that:

A. Fe_{COOK} would be positively associated with:

1. Type 2 diabetes mellitus after controlling for age, BMI, alcohol consumption, smoking status physical activity and micronutrient status.
2. Metabolic syndrome after controlling for age, BMI, alcohol consumption, smoking status physical activity and micronutrient status.
3. Non-alcoholic fatty liver disease after controlling for age, BMI, alcohol consumption, smoking status physical activity and micronutrient status.
4. Hepatic fibrosis, developed by Angulo et al. and defined by the Non-alcoholic fatty liver disease Fibrosis Score (NFS) after controlling for age, BMI, alcohol consumption, smoking status physical activity and micronutrient status.
5. Insulin resistance after controlling for age, BMI, alcohol consumption, smoking status physical activity and micronutrient status.

CHAPTER 2: OVERVIEW OF IRON METABOLISM AND IRON STATUS IN HUMAN POPULATIONS

As an essential micronutrient, humans require iron for cellular respiration, DNA synthesis, DNA repair, as a cofactor in biochemical reactions, oxygen transport, endocrine regulation, immunological regulation and bone health. Thus, iron is a major contributor to subcellular, intracellular, and systemic homeostasis due to the transition metal's capacity for redox cycling (53), where labile iron can be oxidized from ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) and reduced from ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). Homeostasis of human iron metabolism is achieved at two levels: 1) systemic and 2) cellular. Each will be taken in turn, followed by a summary describing their interaction.

Systemic iron metabolism The regulation of systemic body iron occurs at the level of iron absorption (54). Humans acquire iron in two distinct forms: 1) heme iron contained in meats, poultry, seafood and fish and 2) non-heme iron contained in plant-based foods such as vegetables, fruits, grains, nuts, beans and seeds. The heme iron content of meat varies considerably depending upon the source. Red meat contains 50% - 80% heme iron while the heme iron content of chicken, pork or turkey varies between 25% - 40% (55). Compared to non-heme iron, heme iron is absorbed with five to ten times greater efficiency (56) and its absorption is unaffected by current body iron stores (57). Moreover, absorption of heme iron is independent of duodenal pH (58) and is

relatively unaffected by common inhibitors of iron absorption such as phytate and polyphenols (59 Abbaspour et al.). In contrast, the bioavailability of non-heme iron is much less than that of heme iron (5-12%) (60) and its absorption is affected by current body iron stores (61) as well as dietary factors which can either promote or inhibit its absorption (62-64). Despite its lower bioavailability, however, non-heme iron appears to contribute more to human iron nutrition than does heme iron in developed nations (65).

Heme iron is absorbed from the duodenum in the proximal intestine by heme-carrier protein-1 (HCP1); also known as solute carrier family 48 member 1 (SLC48A1)) (66), whereupon it enters the cytosol of the enterocyte and is degraded by hemoxygenase 1 (HOX1) to abstract the central ferrous iron ion from the heme group contained within the porphyrin moiety. In addition to heme iron intake, factors such as hypoxia and iron-regulatory proteins (IRPs) serve to upregulate HCP1 mRNA expression (67). Cytosolic (soluble) ferrous iron in the enterocyte is oxidized to (insoluble) ferric iron by hephaestin, a multicopper oxidase localized mainly within the basolateral membrane of enterocytes (68) which loads oxidized iron onto transferrin, which serves as a siderophore. Briefly, a siderophore (e.g., Greek for "iron carrier") is any small iron-chelating protein or compound which exhibits high-affinity for binding iron (69). Transferrin can bind up to two atoms of ferric iron (70) Hephaestin then works in concert with ferroportin to export transferrin into circulation (68,70) for utilization in the above mentioned physiological processes. The relationship between hepahestin and ferroportin is noteworthy in iron metabolism because under normal physiological conditions, hephaestin is down regulated when body iron is replete and if hepaestin activity is abolished, ferroportin is degraded (71).

In contrast to heme iron metabolism, non-heme iron metabolism is subject to tighter systemic regulation (e.g., non-heme iron metabolism requires an acidic duodenal pH for optimal absorption) (72). Following intake of non-heme iron, duodenal cytochrome B (DcytB), also known as cytochrome b reductase 1 (Cybrd1), a ferrireductase localized within the enterocyte apical membrane, reduces ferric iron to ferrous iron. Divalent metal transporter 1 (DMT1, also known as Nramp2) then transports ferrous iron into cell. DMT1 is expressed at the brush border of duodenal enterocytes and within intracellular endosomes of erythroid precursors (72) and is activated only in an acidic environment. In iron deficiency, DMT1 is upregulated and localizes at both the microvilli and cytoplasm. When body iron is replete, DMT1 is downregulated and localized to the cytoplasm. Similar to the above-mentioned step during heme iron metabolism, hephaestin then oxidizes ferrous iron to ferric iron and works in concert with ferroportin to regulate the efficiency of cellular iron efflux and loads oxidized (i.e., ferric) iron onto transferrin. In cells apart from enterocytes, ceruloplasmin (another multicopper oxidase enzyme synthesized and secreted by the liver with 50% homology to hephaestin) (73) regulates cellular iron export though the mechanism is not yet completely understood (74). Normally, transferrin in circulation is 25-35% saturated with ferric iron ions at a pH of 7.4 (75). Each transferrin siderophore then competitively binds transferrin receptors on target cells with *HFE* protein, which has been shown to function as an iron switch/sensor. In conditions such as hereditary hemochromatosis (HH), a mutation in the HFE gene (i.e., rs1800562: C282Y) leads to misfolded *HFE* proteins which aggregate in the Golgi complex and are thus not available to competitively bind transferrin receptors. Carriers of this mutation experience increased iron absorption due to a lack of this protein

sensor coupled with chronically suppressed hepcidin expression (discussed subsequently) (76). Under normal physiologic conditions, however, transferrin siderophores compete with *HFE* proteins and upon binding with transferrin receptor 1 (TFR1) or transferrin receptor 2 (TFR2) (and to a lesser extent glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) (77-79), transferrin is endocytosed. It should be noted here that studies have reported that TFR1 has ~ 30-fold higher binding affinity for transferrin-bound iron and thus plays a larger role in receptor-mediated iron uptake into the cell (80, 81). Within the low pH of the endosomal compartment iron dissociates from transferrin and both the siderophore and receptor are recycled. However, if pH of arterial blood becomes acidic (i.e., pH < 6.5) transferrin loses it iron loading capacity (82).

As labile (i.e., ferrous) iron accumulates within the cell or within systemic circulation, a soluble protein called ferritin is synthesized by nearly all cell types for iron storage. Each ferritin molecule is a nanocage that stores up to 4,500 atoms of iron and is composed of two heteropolymers, a heavy chain and light chain (83). Labile iron is imported through channels that are created between the bonds of the heavy and light chains (84). Ferritin heavy chain conducts ferroxidase activity that is necessary for iron deposition into the nanocage by converting ferrous iron to ferric iron (85). In addition to labile iron, expression of ferritin heavy chain is induced by NF-kB in response to TNF-α and is higher in tissues that require rapid/high utilization of iron (e.g., heart) (86). In contrast, ferritin light chain facilitates iron incorporation onto ferrihydrite (87), which binds iron to the inner wall of the nanocage and has been observed to participate in ferridoxase turnover. Ferritin light chain expression is higher in tissues that require long term storage (e.g., spleen, liver) (88). As ferritin becomes increasingly saturated, excess

iron precipitates around the protein and causes ferritin cages to aggregate. (89) The resulting insoluble protein is called hemosiderin. Once hemosiderin is formed, the protection provided by ferritin is lost and redox cycling begins (90). As such, some authors define iron deficiency and overload on the basis of the hemosiderin to ferritin ratio, where values less than 1 denote increasing iron deficiency and values greater than 1 denote increasing iron overload (91). While appealing as an index of body iron burden, the quantification of hemosiderin is currently not cost-effective, as the only techniques available as of this writing for measuring hemosiderin are liver biopsy or MRI (92, 93).

Regulating the above-mentioned mechanism is a recently discovered acute-phase protein called hepcidin. Hepcidin is expressed primarily in the liver, though some reports indicate that the protein is also expressed by macrophages and adipose tissue (94) and can be detected in urine, bile, ascitic, pleural, or cerebrospinal fluid (95). Hepcidin regulates body iron homeostasis at the level of duodenal cell absorption of dietary iron and macrophage release of iron from recycled erythrocytes, where the latter leukocytes increase ferritin synthesis with increasing hepcidin expression to sequester iron from invading pathogens. Hepcidin's primary role in iron metabolism is in binding ferroportin, the only known cellular iron exporter. Increasing expression of hepcidin protein in serum results in the degradation, internalization, and eventual recycling of the ferroportin receptor on the cell membrane (96). In turn, this results in decreased export of iron from the cell into circulation.

Several studies have demonstrated that hepcidin expression is regulated by systemic iron availability (i.e., transferrin saturation) (97), hepatic iron stores (i.e., feedback loops between iron sensing proteins such as hemojuvelin (HJV), *HFE* and

transferrin receptor) (98), erythropoietic activity (i.e., which increases in response to hepcidin suppression) (99), hypoxia (i.e., hepcidin expression is suppressed by hypoxia) and inflammatory/infectious states and induced by IL6, IL1β, bone morphogenic protein-2 (BMP-2) and LPS) (100, 101).

Given that less than 10% of daily iron needs are met by intestinal absorption, the remaining iron requirements are fulfilled by macrophages that recycle intracellular iron (102). Macrophages phagocytose senescent or damaged erythrocytes and catabolize them via HOX1 which abstracts the central ferrous iron ion from the heme group contained within the porphyrin moiety after heme is broken down into biliverdin (103) Natural resistance-associated macrophage protein 1 (NRAMP1), a divalent metal transporter homologous to DMT1, is expressed within the phagolysosomal membrane of the macrophage and participates in iron export from phagocytic vesicles (104). Export of ferrous iron from macrophages then occurs via ferroportin, where it is oxidized to ferric iron by ceruloplasmin and bound to transferrin (105). Reflecting its crucial role in systemic iron balance, ferroportin expression in macrophages is regulated at the level of 1) ferroportin transcription induced by erythophagocytosis and heme iron, 2) translation by the IRE/IRP system and 3) protein stability via hepcidin (106).

Cellular iron metabolism   Iron homeostasis at this level of regulation is maintained by specific DNA binding proteins and sequence motifs called iron-regulatory proteins (IRPs) and iron-responsive elements (IREs), respectively. IREs are located in untranslated regions of genes which encode mRNAs that produce proteins involved in iron uptake, storage, utilization and export (70). The binding of IRPs to IREs suppresses the assembly and translation complex involved in ferritin and ferroportin mRNA

synthesis and increases transferrin receptor and DMT1 mRNA expression (70). When iron concentrations are high IRPs are degraded or inactivated causing a downregulation of serum transferrin receptor. IRP1 (also known as ACO1) is activated by iron deficiency (70) and loses affinity for IREs during iron replete status. IRP1 also exhibits aconitase activity. IRP2 (also known as IREB2) is activated by iron deficiency and degraded in conditions of iron excess and does not demonstrate aconitase activity (107, 108).

Iron status in human populations The following sections are an overview of the distribution of iron in the human body, the observed iron status and intake in human populations and a summary of trends and exposures (i.e., dietary and otherwise) which contribute to body iron stores. When data permits, iron status in males and females (i.e., pre-menopausal (nonpregnant), pre-menopausal (pregnant), post-menopausal) are presented separately and summaries are stratified by age and ethnicity.

The total amount of body iron in free-living apparently healthy adults is maintained at a level of about four grams which is distributed in proteins and serum (109, 110). One-to-two mg of iron per day is absorbed from the diet and is balanced by the excretion of 1-2 mg of iron per day via sloughing of intestinal epithelial cells, desquamation of skin and urinary cells, perspiration and fecal transit (109, 110). Total body iron in males typically ranges from 3.5 to 5 grams (109, 110) (~55 mg/kg) and in females ranges from 2 to 3.5 grams (109, 110) (~45 mg/kg). The distribution of body iron in both males and females is quite similar, where most of the metal is contained within hemoglobin (60%), myoglobin and enzymes (10%), ferritin and hemosiderin (20%), bone marrow (10%) and transferrin (~1%) (109, 110). In line with the reported distribution of hemoglobin, the average adult has 4 pints of blood and each pint (i.e., 1/2 liter) of blood

contains 250 mg of iron (109, 110). At ten pints (i.e., 5 liters), the human body therefore contains ~ 2.5 grams of iron in circulation (i.e., 62.5%).

Several studies have shown that indices of iron status vary with gender, age, ethnicity and education (51, 111-114). Surveys of body iron status in populations have consistently demonstrated that males, regardless of ethnicity, begin to accumulate iron in their early 20s and by age 40 have approximately four-fold as much body iron as age-matched females who have not yet proceeded through natural or surgical menopause (112). Thus, compared to males, iron status in females can be affected by factors such as age at menarche, parity, oral contraceptive use and/or age at menopause (115). However, once females reach menopause body iron stores tend to increase and eventually converge with those of males by the 7th decade of life (112). Table 5 contains common iron parameters with reference ranges.

It is hypothesized that blood loss due to regular menstruation should decrease iron stores in females with each menstrual cycle and that an earlier age at menarche should equate to lower iron stores over time. Using data obtained from NHANES 1999-2006, however, Miller reported that self-reported age at menarche was not statistically associated with iron stores at the time of study participation ($P = 0.31$) (115). To my knowledge, this was the only survey to directly examine this hypothesis and replication is warranted before inferences can be made.

Though the number of studies which have examined the association between parity and iron stores is small, two separate population-based studies have nonetheless thus far confirmed the hypothesis that increasing parity is associated with decreased body

iron (116, 117). Thus, these results need to be confirmed by more independent investigators.

The effect of oral contraceptive (OC) use on body iron appears to be related to the formulation. Most OC formulations are composed of synthetic estrogen and progestin, hormones which inhibit the synthesis of follicle-stimulating hormone and leutinizing hormone. These hormones facilitate menstruation and ovum release from the ovaries. Thus, blocking their production via OC use should result in decreased iron loss (118). Frassinelli et al. assessed the effect of an oral formulation on iron parameters in a group of females (n = 46) over the course of two years and observed increased serum iron, transferrin, mean corpuscular volume (MCV), ferritin and decreased TIBC compared to another group of females (n = 71) who never self-administered oral contraceptives (119). The results suggest that OC use does indeed increase body iron. These observations were replicated in a separate investigation conducted by Milman et al. (117) and in a large, population-based study using data from NHANES 1999-2006 (115).

In the U.S., pregnant females often develop transient anemia during the second and third trimesters due to hemodilution and iron loss to the developing fetus. Thus, the prevalence of iron-deficiency anemia in U.S. pregnant females during the second and third trimester has been estimated at 18% (120). Disparities in this estimate are evident, however, when stratifying by ethnicity. Hispanic and African American pregnant females are consistently observed to develop iron-deficiency anemia at higher rates than Caucasian females. Moreover, the disparity persists among nonpregnant African American and Caucasian females, where hemoglobin values among females in the latter ethnic group are higher compared to those of the former (112).

Compared to the adult reference ranges established by the CDC, both Caucasian and African American nonpregnant females have low mean hemoglobin values. According to Cook et al., however, “an estimated 2.6% of premenopausal and 1.9% of postmenopausal females (and 0.2% of males) are iron deficient in the U.S.” (121, p.729) If one extrapolates these data to the population-level, this estimate is quantitatively different than that reported by the CDC, where about 3 million rather than 5 million females would be considered iron-deficient (109). Results published by Cogswell et al. using females participating in NHANES 2003-2004 are congruent with these extrapolations. Cogswell and company estimated the prevalence of iron-deficiency among a sample of 3,742 nonpregnant females using an algorithm developed by Cook et al. for the quantification of body iron and compared these prevalence rates to those generated using a standard model for estimating iron-deficiency (51). Similar to the discrepancy in the prevalence estimates above, the equation developed by Cook et al. “produced lower estimates of iron-deficiency, better predicted anemia and was less affected by inflammation” than the standard model (51, p.1,334). Thus, the discrepancy between CDC estimates and those produced by the algorithm developed by Cook et al. most likely arise due to the difference in hemoglobin reference ranges utilized during the times when incidence was calculated and the fact that national surveys did not take into account the symptoms of iron-deficiency apart from hemoglobin values. In other words, the CDC appears to have used hemoglobin values alone in the determination of iron deficiency anemia. According to Moon, the presence of iron-deficiency anemia is not that common in developed nations (109). For instance, iron-deficiency due to menstruation is not the antecedent of iron deficiency anemia in most females (109). Although

hemoglobin levels and transferrin saturation may temporarily decrease during menstruation, dedicated compensatory mechanisms in the human body insure the absorption of more iron from the diet (109). Therefore, if iron-deficiency anemia does temporarily manifest during the menstrual cycle, it appears to be efficiently counteracted by increased iron absorption (109). This leads one to hypothesize that the iron status among females in developed nations has perhaps been estimated incorrectly.

Using data obtained from participants in the Framingham Heart study and sex-specific cutoffs for defining high iron stores (i.e., ferritin > 300 µ/L in males and > 200 µ/L in females), Fleming et al. observed a high prevalence (12.9%) of elevated iron stores and a low prevalence of iron deficiency (122). Milman et al. observed a similar prevalence (i.e., 13%) of high iron stores in a small sample of 85-year old healthy Danes (123). In another healthy, free-living population of 1,332 Danish men aged 4-70 years, Milman et al. (124) observed a high prevalence of elevated iron stores in males comprising two of the oldest age groups (i.e., 20.8% in males in the 60-year old age group and 15.2% in males in the 70-year old age group). The results published by Garry et al. are congruent with these findings, where the authors report that the prevalence of elevated iron stores in free-living elderly males and females in Mexico were 35% and 15%, respectively when defining elevated body iron stores on the basis of ferritin > 200 µ/L (125). In a separate study conducted by Milman et al. which examined iron status among the Danes from 1994 to those who participated in the Dan-Monica survey ten years earlier the authors concluded that the prevalence of all-cause iron deficiency did not change, whereas the prevalence of iron overload increased (126). Moreover, the authors hypothesized that "the 1987 abolition of mandatory iron fortification of flour apparently

had no negative affect on iron status," (126, p.612) stating that members of the population are ultimately accountable for their own primary prevention. Taken together these studies also appear to support the hypothesis that iron stores increase with age and that the prevalence of high iron stores is relatively common among both males and females in developed nations.

As mentioned previously, body iron stores vary as a function of ethnicity. Using data obtained from NHANES III (1988-1994), Zacharski et al. reported that African American males have a higher body iron burden across the lifespan compared to Caucasian and Hispanic American males and that this disparity is amplified with increasing age (112). Gillum observed a similar trend for Hispanic American males using NHANES III data (113). Unfortunately, the latter author did not compare indices of body iron in these participants to those of other ethnic groups.

Zacharski et al. also observed a similar trend among females following menopause. In other words, no difference in body iron stores among females of childbearing age was observed until after the 4th decade of life when body iron in black females rose more rapidly and exceeded those of Caucasian and Hispanic American females. However, as with most studies assessing body iron, the authors employed ferritin as a proxy for iron stores and did not control for inflammation. Thus, the observed differences in body iron burden across ethnicities by these authors could have been due to the anemia of chronic disease. The results from a more recent study using NHANES data from 2003-2006 are discordant with those of Zacharski et al. among females aged 20-49 years. While the latter authors did not observe disparities in body iron burden among females of childbearing age, the results of Pfeiffer et al. demonstrate higher body iron

stores among Caucasian females followed by Hispanic American and African American females (114). The group used the algorithm developed by Cook et al. in the estimation of iron stores. More research is needed before inferences can be made on these results.

In a follow-up study examining dietary factors associated with high iron stores in the Framingham cohort, Fleming et al. concluded that intakes of highly bioavailable forms of iron (e.g., supplemental iron and heme iron from red meat) and of fruit with vitamin C (i.e., an enhancer of iron absorption) promote high iron stores, whereas foods containing phytates (e.g., whole grains) decrease these stores (127). The results from another population-based study are congruent with these findings. In a study published using participants in NHANES III, the proportion of iron supplement users whose intake was at or below the recommended dietary allowance (RDA) was 18.1% in males aged 19-30 years (n = 773), 21.7% in males aged > 31 years (n = 2,235), 59.3% in premenopausal females (n = 1,553) and 22.5% in postmenopausal females (n = 1,387). From these data, one can infer that over 81% of males aged 18-30 years, 78% of males aged 31 years and older, 40% of premenopausal females and 77% of postmenopausal females consume iron supplements in quantities greater than those recommended by the RDA. Among users with very high supplemental iron intakes, the authors report that males aged 19-30 years who consumed > 4 times the RDA was 12% and 12.7% in males > 31 years. The proportion of premenopausal and postmenopausal females who consumed > 4 times the RDA was 4.2% and 8.2%, respectively (128). The results published by Garry et al. are in alignment with these findings. In their study of free-living elderly males and females living in Mexico the authors reported that participants consuming > 18mg (i.e., roughly twice the RDA) had iron stores that were 65 mg higher

than participants who did not consume iron supplements (125). The current RDA for dietary iron and supplements combined in females of childbearing age is 18 mg/day. For pregnant females, the RDA is 27 mg/day and during lactation it is 10 mg/day. For all other adults, the RDA is 8 mg/day (129). Thus, it is not surprising that much of the population in developed nations is iron-replete, where the reported median dietary iron intake for men is 16-18 mg/day and 12 mg/day for postmenopausal females (109, 128).

As mentioned previously, the rate of iron absorption depends on a variety of factors, such as body iron stores, rate of erythropoiesis, hemoglobin concentration and oxygen concentration. Several studies have demonstrated that the average rates of iron absorption in males and nonpregnant females is 15% of total dietary iron while pregnant females absorb approximately 17% of ingested iron. According to the Food and Nutrition Board, roughly half of the total amount of dietary iron ingested by the human population originates in fortified foods (i.e., flour, pastas, bread, cereals) (109). This is not surprising, given the mandatory fortification of the U.S. food supply with iron beginning in 1941 (109). It has been reported that some cereals contain up to 24 mg of iron per one-cup serving (109) and Abbaspour et al. have demonstrated that the iron content in different varieties of wheat can vary from 25 mg to 56 mg/kg and iron in various strains of rice grains can vary from 7 mg to 23 mg/kg (59).

In addition to fortified foods, body iron can be increased by means of other dietary exposures which increase its absorption. For instance, consumption of sugar has been shown to increase non-heme iron absorption by four-fold (62). Other dietary exposures which increase intestinal iron absorption are beta carotene, cauliflower, tomatoes, kiwifruit and oranges (130). Ascorbic acid (i.e., vitamin C) induces a change in

the redox potential of non-heme iron, shifting the valence from ferric iron to ferrous iron (109, 131). As mentioned earlier in the current chapter, the latter form is more soluble and 40-50% of iron ingested concomitantly with ascorbic acid is readily absorbed in the duodenum compared to the average rate of absorption of 15% or less (131). Moreover, concomitant consumption of red meat with non-heme iron can result in a significant increase in iron absorption and it has been reported that one gram of red meat intake has an enhancing effect on non-heme absorption equivalent to that of one milligram of ascorbic acid (110). Given that the heme iron content of the typical Western diet averages ~10-15% (i.e., from red meats, processed meats, poultry and fish), it doesn't appear as though heme iron is an independent contributor to total body iron stores. In other words, both heme iron as well as nonheme iron, in addition to exposures which increase and/or decrease iron absorption are all factors which must be accounted for when estimating total body iron stores.

Exposures not typically found in the diet which nonetheless increase iron absorption include alcohol and smoking. Approximately 20-30% of heavy drinkers acquire up to twice the amount of dietary iron as do abstainers and light drinkers (132, 133) because alcohol inhibits delta-amino-levulinic acid dehydratase, a key synthetase in porphyrin biogenesis and, ultimately, hemoglobin levels (134). Alcohol also increases non-heme iron absorption due to increasing gastric acid production, where the increased acidity precipitates iron ions in solution as they reach the duodenum where absorption into bloodstream occurs (134). In addition, alcohol decreases hepcidin synthesis and increases expression of DMT1 in the intestine, resulting in increased iron absorption (135). A typical cigarette contains approximately 84 micrograms of iron per gram of

tobacco (110, 136) Given that a cigarette contains approximately one gram of tobacco, a one-pack-a-day smoker can inhale up to 1.68 milligrams of iron per day. Moreover, some pipe tobaccos contain over 400 micrograms of iron per gram of tobacco (110, 136). Consistent with these statistics, alveolar macrophage iron content has been shown to be 67% higher in smokers compared to nonsmokers (137) and is positively correlated with number of cigarettes smoked (138).

Iron loss can occur through several routes, such as blood loss through donation and phlebotomy, menstruation and child birth. Blood donation and phlebotomy typically removes one pint (~ 475 cc) of blood and this equates to 0.25 grams (250 mg) of iron (109, 110). Blood is also lost during menstruation. The average period lasts anywhere from two to five days and blood loss during this time is estimated to range from 60 milliliters (~ 4 tbs) for a light period to 250 milliliters (~ 1 cup; half of a pint; 125 mg of iron) for a heavy period (110). On average, however, about one pint of blood is lost annually during menstruation (110), which is equivalent to the amount of iron loss during blood donation/phlebotomy. Each child birth results in a loss of blood that is equivalent to the amount lost during a heavy period (110). Other factors which result in loss of iron include physical activity (i.e., iron is lost through perspiration and sloughing of the gastrointestinal tract), ulcers, trauma, hemodialysis, surgical procedures, colon cancer, uterine cancer, intestinal polyps, hemorrhoids and intravascular hemolysis (109). When hemolysis occurs, hemoglobin and heme iron are reported to be 1) scavenged by haptoglobin and hemopexin, respectively, 2) rapidly cleared from circulation and stored in ferritin, 3) recycled by macrophages or 4) lost by hemoglobinuria and hemosiderinuria, respectively (109, 110).

Notwithstanding the above-mentioned routes of blood loss, body iron stores can be reduced at the level of absorption. Dietary exposures which decrease iron absorption are fiber, soy protein, inositol phosphate-6 (IP6) (i.e., phytates/phytic acid) such as wheat bran, rice bran, whole wheat, corn, peas, rye, oats, brown rice, nuts and legumes (130, 131) as well as oxalates such as spinach, bran flakes, rhubarb and beets (130). In addition, polyphenols such as flavonoids, phenolic acids, phenolic alcohols, stilbenes and lignans are ring structures that contain more than one hydroxyl group which bind non-heme iron and render it unavailable for absorption (131). Calcium has been shown to inhibit non-heme iron absorption (and heme-iron, to a lesser extent) and ovotransferrin (i.e., ovalbumin) contained in eggs exhibits an iron-binding affinity similar to that of lactoferrin (110). Lactoferrin (found in bodily fluids such as perspiration, tears, seminal fluid, vaginal fluid, breast milk and to a lesser extent in saliva) (110) binds ferric iron with 240-times greater affinity than transferrin (110) and deprives microorganisms (e.g., bacteria, fungi) access to growth medium. In contrast to transferrin, however, iron that is sequestered by lactoferrin is not used by healthy cells (i.e., the sole function of lactoferrin is to bind excess iron) (109, 110). Consistent with these observations, it has been reported that one boiled egg can reduce absorption of iron in a meal by as much as 28%. (109). Finally, medications such as acid reducers (e.g., omeprazole (Prilosec), pantoprazole (Protonix)), Imipramine (i.e., an antidepressant) and aspirin have also been shown to reduce iron absorption. According to Weinberg, gastrointestinal microbleeding induced by aspirin “results in the loss of iron comparable to that of menstrual blood loss.” (110).

CHAPTER 3: IRON IN HUMAN HEALTH AND DISEASE

Iron in human health The importance of iron in human health was evident as early as the 17th century when iron was used to treat chlorosis, a condition which results from iron deficiency. However, it was not until 1932 when it was demonstrated that iron was required for hemoglobin synthesis that the metal began to become a fixture in the research community (109).

Functional dependencies on iron As mentioned in chapter 2, humans require iron for cellular respiration, DNA synthesis, DNA repair, as a cofactor in biochemical reactions, oxygen transport, endocrine regulation, thermoregulation and bone health. Three out of the four redox cofactors (i.e., complexes) in the electron transport chain and the electron carrier cytochrome c contain hemes and iron-sulfur clusters, respectively. All contain iron ions whose redox state serves to accept or donate electrons to keep the chain operational where ferrous iron acts as an electron donor and ferric iron acts as an electron acceptor (139).

With respect to DNA synthesis and repair, the activity of ribonucleotide reductase, the rate limiting enzyme involved in the conversion of ribonucleotides to deoxyribonucleotides is iron dependent (140). Iron is also vital for the enzymatic activity in xenobiotic (e.g., plant alkaloids, fungal toxins) and medication metabolism. All cytochrome P450 (CYP450) enzymes contain iron in their active site where the redox potential of iron is used to catalyze the oxidation (i.e., activation) of most medications

(141-144). These enzymes are expressed in every tissue except skeletal muscle and erythrocytes (142). The reactions catalyzed by CYP450 enzymes begin with the abstraction of hydrogen from NADPH by cytochrome P450 reductase (142). The hydrogen is then used by CYP450s to reduce one of the two atoms of molecular oxygen to water. The other oxygen atom is retained in a highly reactive form, which in turn is used to force subsequent catalytic reactions upon the substrate (142-144).

The enzymatic activities of aconitase (i.e., a regulatory protein), nicotinamide adenine dinucleotide phosphate (NADPH) oxidoreductase and myeloperoxidase are dependent upon iron as a cofactor. In addition, catalase is an enzyme which uses iron as an electron donor and participates in the prevention of hydrogen peroxide (H_2O_2) induced cellular damage by converting the compound to water and molecular oxygen (145). Xanthine oxidase contains riboflavin (i.e., vitamin B2), molybdenum and ferrous iron in its active site (146). Finally, iron appears to be a negative regulator of nitric oxide (NO) synthase (147). Conversely, NO is a negative regulator of iron, whereby increased NO decreases labile iron by increasing ferritin mRNA expression and ferritin synthesis (148) and NO has been observed to be up-regulated in iron deficiency anemia (149).

The genesis of oxygen transport occurs upstream within the bone marrow beginning with the synthesis of erythrocytes (i.e., erythropoiesis) and requires 25-30 mg of iron per day. The iron is obtained from reticuloendothelial macrophages which phagocytose and catabolize 20 mL of senescent erythrocytes each day resulting in the 25-30 mg of required iron (150). Erythroid regulation of iron metabolism is controlled by erythroferrone (ERFE), a newly identified hormone produced by erythroblasts in response to erythropoietin which negatively mediates hepcidin expression during iron-

deficient states. Briefly, the kidney acts as a sensor of oxygen levels in circulating erythrocytes. When oxygen levels decrease, erythropoietin is synthesized by interstitial fibroblasts within the renal medulla (151), which is released into circulation and upregulates erythroblast formation in the bone marrow. In turn, erythroblasts express ERFE, which lowers serum hepcidin and increases iron absorption (151). Thus, iron utilization for erythropoiesis increases after stimulation by erythropoietin or in response to hypoxemia (152). Conversely, inhibitors of erythropoiesis induce a rise in serum and tissue iron levels (153).

Iron affects endocrine signaling in myriad ways. For example, increases in adipocyte ferritin synthesis has been shown to reduce adiponectin expression and increases insulin resistance (154, 155). In addition, iron and leptin appear to negatively regulate one another, as increases in leptin induce hepcidin expression (which decreases iron absorption) (156). Paradoxically, increases in iron are monotonically associated with decreases in leptin. For example, in a 2015 cross-sectional study conducted by Gao et al. using data from a cohort of 76 individuals, the authors sought to examine the relationship between serum ferritin and leptin and found a significant negative association between the two analytes ($r = -0.53$, $P < 0.0001$). Moreover, to extend these findings, the authors conducted an animal study using C57BL6/J mice, where the animals were fed one of two diets: 1) a high-iron diet (i.e., 2,000 mg/kg, N = 36) or 2) a low-normal iron diet (i.e., 35 mg/kg, N = 36) for 2 months. At the end of the study, Gao et al. found that serum leptin decreased with increasing dietary iron ($r = -0.26$, $P < 0.01$) where mice in the high-iron group had 42% lower serum leptin concentrations compared to mice in the low-normal iron group (157).

The relationships between sex hormones and body iron appear to be quite complex. For example, testosterone increases expression of erythropoietin, which decreases hepcidin expression to sequester iron for erythropoiesis (158). It has also been shown that testosterone directly suppresses hepcidin expression and increases iron absorption independent of erythropoietin signaling (159). Consistent with these results, Liu et al. reported that serum ferritin levels are inversely associated with total testosterone, sex-hormone binding globulin (SHBG) and free testosterone (160) and Winters et al. have demonstrated that SHBG is inversely related to insulin resistance (161) and metabolic syndrome (162). Yang et al. have recently shown that E2 contained in oral contraceptives suppresses hepcidin expression both *in vitro* and *in vivo* and increases iron absorption by binding to an estrogen response element in the hepcidin promoter gene. (163, 164). In an animal study using newly hatched White Leghorn chicks, E2 administration decreased total iron binding capacity (TIBC, i.e., the ratio of serum iron to transferrin saturation) in serum by ~ 20% from levels in a control of group of animals (n = 4) irrespective of iron content of diet and raised non-heme iron content of livers of animals in the experimental group (n = 4) by 40-50% over that of the control group irrespective of iron content of diet. (165). More alarming is that redox cycling of estrogen metabolites has been reported to release ferric iron from ferritin which in turn generates hydroxyl radicals via the Fenton reaction (discussed in greater detail below) (166).

Human and animal studies have both reported links between excess body iron and decreased bone mineral density (BMD), lower serum osteocalcin, delays in bone mineralization, lower rates of bone formation and lower osteoblast numbers (167).

Individuals with beta-thalassemia, for example, consistently exhibit low levels of circulating osteocalcin (168) and osteoblast cells express transferrin receptor and ferritin throughout differentiation *in vitro*, suggesting that iron is important in osteoblast maturation and function (167). Iron suppresses osteoblast maturation and function in a dose-dependent and time-dependent manner (167) in cells isolated from primary fetal rat calvaria and it is hypothesized that that the effect is mediated by ferritin and its ferridoxase activity (169). As previously mentioned, ferritin light chain participates in ferridoxase turnover and studies have reported that ferritin light chain expression is normally high in osteoblasts (167). Thus, as intracellular concentrations of iron increase, ferridoxase turnover activity of ferritin light chain may diminish, leading to an increased number of immature osteoblasts. Moreover, cell line studies where DMT1 is mutated to increase iron absorption demonstrate that the ensuing iron overload leads to increased osteoblast autophagy and apoptosis (170). Taken together, it appears as though the effect of increased iron on bone health results in premature cell death and/or proliferation of immature osteoblasts. Finally, one group of authors have published data which indicates that serum ferritin is inversely correlated with osteocalcin and osteocalcin is associated with glucose and insulin metabolism by exerting a direct effect on beta cells via stimulating insulin production and enhancing adiponectin production from adipocytes (171). Thus, these researchers have recently developed a hypothesis which links high body iron to low osteocalcin, low adiponectin and increased insulin resistance (172).

Iron has been implicated in the body's ability to thermoregulate for quite some time. For example, several authors have reported that iron deficiency leads to impaired thermoregulation in response to acute cold exposure (173-176). Conversely, it has also

been hypothesized that 'hot-flashes' experienced by post-menopausal females are directly related to increases in body iron following the onset of menopause (177).

Iron in human disease An approach often employed by epidemiologists to implicate a given exposure in the development of disease is to demonstrate that the disease has a genetic or heritable component. Most diseases of iron overload result from loss of function mutations (e.g., hereditary hemochromatosis), resulting in suppressed hepcidin production. To this end, the following iron loading conditions have all been studied extensively and linked to adverse health outcomes: hereditary hemochromatosis, TFR2-related hemochromatosis, ferroportin disease, juvenile hemochromatosis (type 1 and 2), beta thalassemia, sickle cell anemia, X-linked sideroblastic anemia, pyruvate-kinase deficiency, hereditary spherocytosis, Fanconi anemia, Diamond-Blackfan anemia, chronic renal failure, Friedreich ataxia, hereditary atransferrinemia and hereditary aceruloplasminemia (178-181). Tables 6a-6e highlight each iron loading disorder, genetic mutation, genetic locus, mode of inheritance, mechanism, affected iron parameter and disease association(s)/clinical feature(s). For convenience, each disorder is categorized by subtype.

Iron in infectious disease Nearly all pathogenic microorganisms need iron for survival, growth and multiplication (110). Under normal physiologic conditions an immunological challenge results in an increased expression of interleukin-6 (IL6). In turn, hepcidin mRNA expression is reduced and interferon gamma (INF-γ) is induced. This results in the halt of export of iron from cells via degradation of ferroportin and a reduction of transferrin receptor expression, which enables invaded cells (e.g., macrophages) to starve invading pathogens. Several infectious microorganisms such as

Mycobacterium tuberculosis, Candida albicans, Borrelia burgdorferi, Staphylococcus aureus, Pseudomonas aeruginosa, Helicobacter pylori, Salmonella, Toxoplasma gondii directly induce hepcidin expression via pathogen associated molecular patterns (PAMPs), which also prompts the body to withhold iron and trigger the innate immune response via activation of toll-like receptors (182). Studies have shown that circulating hepcidin binds to TLR2, TLR3, TLR4, TLR7, TLR8, TLR9 and TLR10 (183). Pathogens, however, have evolved at least four strategies to acquire iron from hosts: 1) hemolysis (e.g., *Streptococcus pneumoniae, Staphylococcus aureus*), 2) transferrin or lactoferrin scavenging (e.g., *Neisseria meningitis* (i.e., transferrin scavenging), *Helicobacter pylori* (i.e., lactoferrin scavenging), *Vibrio vulnificus* (i.e., transferrin scavenging), 3) siderphores (e.g., *Escherichia coli, Listeria monocytogenes, Haemophilus influenzae* (183)), *Neisseria meningitidis* (183) or 4) target, infect and utilize intracellular macrophage iron (e.g., *Coxiellaceae*, *Francisella tularensis*, *Legionnaires pneumoniae, Mycobacterium tuberculosis*). Moreover, hepatitis C virus has been shown to increase hepatic iron level in Huh7 and Huh7.5 cell lines by suppressing hepcidin expression (184) and five iron-dependent and iron-regulated steps are required for the replication of the human immunodeficiency virus (HIV) (185).

Increases in body iron burden have been linked to the suppression of the complement system (186) and have been observed to have adverse effects on major aspects of macrophage/monocyte function such as decreasing immune and nonimmune phagocytosis, decreasing chemotaxis, altering cytokine production and release as well as decreasing antigen processing for subsequent presentation to B- and T- lymphocytes (186). Increased iron concentration in macrophages reduces their response to activation

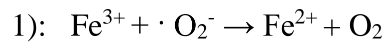
by interferon-gamma (INF-γ) and diminishes their antimicrobial toxicity. Moreover, cell-line studies have demonstrated that addition of iron to culture results in decreased antibody- (Ab) mediated and mitogen-stimulated phagocytosis by macrophages and monocytes (186). Conversely, orally-administered lactoferrin has been shown to restore humoral immunity in immunocompromised mice (187). It has been hypothesized that the above associations between iron and macrophage/monocyte function is due the ability of iron to negatively regulate NO synthesis. Given that NO is integral for macrophage cytotoxicity (188), this hypothesis warrants further investigation.

Increases in body iron have also been implicated in alterations in T-lymphocyte subsets. For example, individuals with high iron stores demonstrate a reduction in circulating interleukin-2 (IL2) and subsequent impairment of the generation and activity of CD8+ cytotoxic T lymphocytes (CTLs) (186, 189) and natural killer cell (NKC) activation and proliferation. Moreover, these lymphocytes in individuals with increased body iron exhibit decreases in proliferative capacity, numbers and activity of helper (CD4) T cells (186) by increasing expression of IL-4, IL-10, and decreasing levels of IL-12 and INF-γ. Thus, increases in iron appear to polarize helper T-cell subsets into Th2 effector cell subtypes which are implicated in reduced immunological surveillance and anergy (183). This is thought to arise via the same mechanism implicated in macrophage anergy whereby iron antagonizes NO, which decreases Th1 proliferation (190) and polarizes the Th2 response. In turn, increased Th2 production of IL4 induces a suppression of INF-γ and this culminates in the activation of transferrin receptor mRNA synthesis which could lead to further increases in body iron. Clinical studies which support these *in vitro* results have shown that iron demonstrates an antagonistic effect for

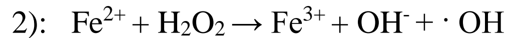
individuals undergoing interferon therapy (191) and that treatment response is restored upon iron chelation therapy (191). In line with these observations, body iron also appears to have an effect on interferon therapy. The higher iron content of the liver, the less likely it is that a patient will respond to treatment (109). Related to this, Alexander et al. (192), performed weekly phlebotomies on 18 subjects with chronic hepatitis C infection who did not respond to initial interferon therapy. Phlebotomy continued until serum ferritin was < 50 ng/mL and the authors observed that "iron depletion was associated with a response rate of 22% in those who did not initially respond to monotherapy." Taken together, it appears that the effects of iron on immunological regulation is related to immunological defense and surveillance.

Iron in chronic disease As a dietary exposure, iron appears to have a curvilinear relationship with human health and disease, as both severe iron-deficiency and varying degrees of iron excess have been implicated in the pathogenesis of several adverse health outcomes. As mentioned, the idea that excess iron could be potentially toxic to humans was first suggested by Jerome Sullivan in 1981 in his canonical article "Iron and the sex difference in heart disease risk" (36). In it, Sullivan hypothesized that blood loss via menstruation is the primary factor that protects premenopausal females from ischemic heart disease. A few years later in 1984, Halliwell and Gutteride demonstrated iron's toxicity via oxygen radical generation in biological systems (193). Finally, in 1992 with Lauffer's publication of Iron and Human Disease, iron became widely known as a bio-metal that accumulates with increasing age (194)

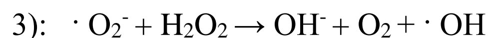
Though iron is an essential micronutrient required for survival it is also a transition metal. Thus, iron can exert a detrimental effect on cellular, organ, and systemic homeostasis via the Fenton Haber-Weiss reaction, which can be summarized as follows:



$$1): \quad Fe^{3+} + \cdot O_2^- \rightarrow Fe^{2+} + O_2$$



$$2): \quad Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$$



$$3): \quad \cdot O_2^- + H_2O_2 \rightarrow OH^- + O_2 + \cdot OH$$

High levels of insoluble iron can react with superoxide radicals and ferrous iron can react with H_2O_2, initiating the Fenton reaction (reactions 1 and 2). The end-product of these reactions includes the generation of unstable free radicals and oxidized ferric iron (195). Iron is also a key metal that catalyzes the Haber Weiss reaction, which is a secondary reaction to the Fenton reaction (196). A superoxide radical ($\cdot O_2^-$) generated from the Fenton reaction reacts with H_2O_2 which in turn generates a hydroxyl radical (196). Thus, iron is a pro-oxidant and is capable of generating reactive oxygen species (ROS). In turn, ROS contribute to oxidative stress (OS) and inevitably induce lipid peroxidation, endothelial dysfunction, and microsatellite mutations within regions of the genome (197). It is noteworthy to mention that the above reaction is an intracellular phenomenon. Thus, several authors have concluded that increased intracellular iron, in comparison to extracellular iron, has been shown to exhibit greater systemic toxicity and cellular damage.

In developed nations including the Unites States, the seven leading causes of human mortality are cardiovascular disease (e.g., coronary heart disease), cancer, cerebrovascular disease (e.g., stroke), chronic obstructive pulmonary disease (COPD),

accidents, Alzheimer's disease (AD), and diabetes (198). With the exclusion of accidents and diabetes, the ensuing review will examine the effect of iron deficiency and excess on the development of human chronic disease. A review of the effect of iron in the epidemiology of diabetes and other metabolic dysfunction appears in chapter 4.

Iron and cardiovascular disease Cardiovascular disease (CVD) is the leading cause of mortality in the U.S., accounting for 25.4% of total deaths (199). The postulation that iron influences the development of CVD originated with J.L. Sullivan in 1981 when the author published an article in the Lancet examining gender differences in the risk of heart disease (36). Sullivan proposed that the rate difference in incident CVD between men and females was likely due to iron loss through menstruation in premenopausal females. Thus, Sullivan's hypothesis stated that iron excess was a risk factor for the development of CVD, while iron deficiency appeared to convey a protective effect.

The iron-heart hypothesis has received support from *in vitro* models (200, 201), animal studies (200, 201) and epidemiological studies (202-211). For example, in a prospective study in a Finnish population (n = 1,931), Salonen et al (200) demonstrated that serum ferritin concentration was positively associated with risk of myocardial infarction (MI). Compared to men with serum ferritin levels < 200 ng/mL, men with serum ferritin concentrations ≥ 200 ng/mL exhibited more than a 2-fold increased risk of acute MI (200). In a study of 9,920 Canadian men and women, Morrison et al (201) have shown that the risk of fatal MI increases significantly in persons with serum iron concentrations ≥ 175 µg/dL combined with total cholesterol level ≥ 240 mg/dL. Tzonou and colleagues (202) examined the role of dietary iron in the development of coronary heart disease (CHD) in a case-control study in Greece. The study was comprised of 329

cases and 570 controls with no matching on age or gender. Cases were all recruited from the same hospital on the basis of CVD development. Hospital controls were selected on the basis of absence of CVD. The authors demonstrated a positive association between iron intake and CHD among cases > 60 years of age. The odds ratio that the authors reported for each monthly increment of 50 mg of iron was 1.47 (95% CI: 1.02-2.12). The effect of dietary iron was stronger for women (OR for each monthly increment of 50 mg dietary iron: 3.61; 95% CI: 1.45-9.01). Although slightly attenuated when adjusted for total cholesterol and blood pressure, the risk estimates remained significant for both men (OR = 1.36) and women (OR = 3.51). Thus, the results published by Tzonou et al support Sullivan's hypothesis. Findings from van der A, et al (203) support the increased risk of CVD in females with diets high in heme iron. Participants were grouped into quartiles based upon increasing levels of dietary iron. In the study (n = 16,136), the authors found a 65% increase in CVD risk in middle-aged women who consume > 2.27 mg/day heme iron (Q4 vs Q1: HR = 1.65; 95% CI: 1.07-2.54). The significance of the risk estimates did not change when the authors adjusted for total energy intake or total cholesterol intake. Results from the Bruneck Study, however, have shown that serum ferritin is one of strongest predictors for progression of atherosclerosis in the presence of increasing concentrations of LDL cholesterol (204). The authors demonstrated that a 1-SD increase in ferritin concentration at LDL levels of 2.5 mmol, 3.6 mmol, and 4.9 mmol precipitated an increase in progression of atherosclerosis. The reported hazard ratios (HR) for each increasing concentration of LDL were 1.55 (95% CI: 1.30-1.85), 1.77 (95% CI: 1.40-2.24), and 2.05 (95% CI: 1.50-2.80), respectively. These results suggest an interaction between iron and LDL cholesterol. A study conducted by Bazrgar et al (205) supports

this speculation. The authors examined the association between APOE status in patients with thalassemia major and the risk of left ventricular dysfunction. Participants (n = 202) were divided into three groups, based upon level of cardiac impairment (i.e., group 1= no cardiac impairment; group 2 = LV dilation with normal systolic function; group 3 = LV systolic dysfunction). Participants in group 3 were significantly more likely to carry the high risk APOEε4 allele, compared to participants in group 1 or group 2. Briefly, gene products of APOE function as lipid transporters and scavengers of free radicals (205). The locus itself is known to contain three variant alleles: APOEε2, APOEε3, and APOEε4. APOEε2 encodes greater concentrations of mRNA and displays high scavenging activity while APOEε4 encodes lower concentrations of mRNA and has exhibits decreased scavenging activity (205). The scavenging capacity of APOEε3 lies between APOEε2 and APOEε4. Products of the APOE gene thus work to clear oxidized LDL (oxLDL) from systemic circulation (205, 206). It has been well established that ox-LDL is one of the main contributors to atherosclerosis and that iron is a potent oxidizing agent (206). Hence, the synergistic relationship between iron and LDL cholesterol appears biologically plausible. Interestingly, cholesterol lowering medications (i.e., statins) have been shown to reduce serum ferritin levels, offering further support for iron in the iron-heart hypothesis (207).

Genetic studies of CVD have contributed additional insights to risk susceptibility. For example, a prospective cohort study (n = 1,150) conducted by Tuomainen et al (208) demonstrated that heterozygous male carriers of the risk allele (i.e., C282Y) of the HFE gene, a mutation implicated with hereditary hemochromatosis, experience a 2.3-fold increased risk of acute MI ($p = 0.03$). These results were subsequently substantiated in a

study by Roest et al (209), who have shown that female HFE heterozygotes also have an increased risk of mortality from cardiovascular disease (OR = 1.6; 95% CI: 1.1-2.4)

Further supporting the iron-heart hypothesis are studies which demonstrate a decreased risk of CVD among regular blood donors. Another prospective cohort study (n = 2,862) conducted by Salonen et al (210) demonstrated that blood donors experienced fewer CVD events (i.e., acute MI) compared to non-donors (0.7% vs 12.5%, respectively; $p = 0.035$). Results from Ascherio et al (211), however, do not corroborate these findings. The authors argue that healthier individuals are more likely to donate blood and therefore do not constitute a random sample. It should also be noted that the literature offers further discordant support for the iron-heart hypothesis. For example, during a 14-year follow-up study, Sempos et al (212) found no increased risk for CVD with increasing levels of transferrin saturation in a cohort of 4,518 men and women. These results have been confirmed in the Helsinki Heart Study (213) and the Health Professionals' Follow-up Study (214). A retrospective cohort study published from data gathered during the NHANES I Follow-up Study demonstrated a negative association between serum iron concentration, transferrin saturation, and CVD (215). In a controlled-feeding study Derstine and colleagues (216) reported no association between iron status and strong risk factors for cardiovascular disease (i.e., LDL oxidative susceptibility). It is interesting, however, that participants in the aforementioned study were administered food sources containing only non-heme iron (e.g., peanut butter). The literature clearly demonstrates that heme iron from animal sources (202, 203, 217), which is more readily absorbed in the gastrointestinal tract, is more closely linked to the association between

excess iron and cardiovascular disease risk. Derstine et al offered no reasoning for the exclusion of heme iron from each participant's diet.

Taken together, it appears that the iron-heart hypothesis finds support from human studies examining the relationship between excess iron stores (derived from heme-iron intake) and acute or ischemic cardiovascular-related phenomena. Thus, iron in excess appears to exert its effect in initiating or promoting CVD events (218). It should be noted that many markers of iron status (i.e., ferritin and transferrin) are acute-phase reactive proteins often present during infection and inflammation. Studies that have controlled for these related factors by concomitantly measuring serum C-reactive protein (CRP) (219, 220) and α-1 acid glycoprotein (AGP) (220) nevertheless reveal significant associations between excess iron stores and risk of CVD. Given that the aforementioned relationship between iron and CVD appears to be significantly modified in persons with APOEε4 status and/or abnormal lipid profiles (e.g., hyperlipidemia), further research is required to quantify iron's unique contribution to the development of CVD.

Iron and cancer Malignant neoplasms (i.e., cancers) are the second leading cause of mortality in the U.S., accounting for approximately 23.2% of total deaths (198). The relationship between iron excess and cancer has been studied extensively. Multiple disciplines, ranging from genetics to epidemiology have reported upon the toxicity of iron in the human body. Emerging from these investigations is a seemingly coherent communiqué: iron in excess is positively associated with cancer mortality and the initiation or promotion of specific cancers. As mentioned previously, iron is capable of generating ROS, inducing lipid peroxidation, or endorsing microsatellite mutations. The culmination of the preceding events invariably leads to mutations within DNA as well as

organelle, cellular, and tissue damage (221-224). Moreover, forward propagation of these aberrant intracellular states often initiate irreversible mutations in essential DNA repair or tumor suppressor genes. In turn, an entire diabolical cascade of chemical events may ensue, leading to the dysfunction and destruction of mitochondria, organelles, and tissues. It remains unresolved, however, if iron conforms more suitably to the role of tumor initiator or promoter.

Iron does not have carcinogenic properties, per se. Nevertheless, many studies have clearly linked iron to the development of malignancy. For example, using NHANES II data, Wu et al (225) demonstrated a dose-response effect of iron on cancer mortality. An investigation utilizing NHANES I data support the aforementioned results (226). Regarding cancer subtype, iron has been most strongly linked to the development of colorectal cancer (227-239) and breast cancer (240-255). These studies demonstrate that iron-specific parameters derived from study participants are often indicative of iron overload (as opposed to iron-deficiency) in these cancers. Furthering the association between iron excess and cancer are studies demonstrating an association between colorectal and breast cancer with the HFE C282Y mutation (256-262). Iron's association with lung cancer is less clear, however. Four of six investigations establish a point estimate greater than one (263-266). However, only one of the six (263) estimates do not cross unity. Interestingly, iron appears to espouse a dual-role in prostate cancer (267-275). Iron tends to be inversely associated incident prostate cancer (267-271) and positively associated with advanced, aggressive, and fatal prostate cancer (272). The subjects from each of the aforementioned studies were all middle aged and older, rendering it difficult to distinguish iron from other milieu as a tumor initiator. Given the

associations reported in the above investigations, however, regarding iron as a tumor promoter seems plausible. This speculation is further supported by human and animal studies demonstrating a clear association between primary iron overload (i.e., hereditary hemochromatosis) and secondary iron overload with digestive tract and liver cancer (276-283). These sites are of particular interest, given their primary function in iron absorption, metabolism, and storage. Interestingly, tumor cells have been observed to express a high number of transferrin receptors (197), owing to their high rate of proliferation. Thus, it would be a formidable task to abrogate the role of iron in the promotion of malignancy in humans. Moreover, irrespective of initiating events, the alterations observed in iron status and metabolism in the above-mentioned cancers indicate that iron bolsters the aggressiveness and metastases of malignancy.

Although iron is clearly implicated in the development and propagation of colorectal and breast cancer, investigations examining the role of iron in other types of cancer show no such associations. For example, inverse associations between iron status and renal cancer, pancreatic cancer, Barrett’s esophagus, and oral cancer have been reported in several studies (284-286). Nevertheless, these results do not diminish the significance of the role of iron in other tumor subtypes. For example, it is well known that iron chelators have been used in the treatment of certain types of cancer (287-290). Moreover, regular blood donation (291), phlebotomy (291), and anemia (292) are inversely associated with the subsequent development of malignancy.

The association of iron with childhood malignancy has not yet been directly examined. However, one can speculate that iron does indeed appear to be indirectly involved in the development of childhood malignancy. For example, Dorak et al (293)

have noted that the HFE C282Y mutation is a male-specific risk factor for childhood acute lymphoblastic leukemia (ALL). In addition, other studies have teased apart an association between high birth weight (i.e., macrosomia) and childhood leukemia (294-297). Lending support to this association are studies demonstrating a relationship between macrosomic infants of diabetic mothers and childhood cancer (298-301). Moreover, as indicated by Shetty et al in 2008, human and animal studies have shown a positive linear relationship between labile iron concentration and glycated hemoglobin (HbA_1C) (302). In addition, macrosomic infants have also been shown to have increased numbers of hematopoietic stem cells (303), which has been correlated with increased cancer risk.

Iron and cerebrovascular disease Cerebrovascular disease is the third leading cause of mortality in the US, responsible for approximately 5.6% of total deaths (198). However, there is a paucity of literature specific to the association between iron and cerebrovascular disease (i.e., stroke). The available studies report that both iron deficiency and iron excess are risk factors for cerevrovasular disease. For example, a case report by Hartfield, et al (304) indicated that iron deficiency was the only common feature among six infant stroke patients. In a case-control study (15 cases:143 controls) examining the relationship between iron and childhood stroke, Maquire et al (305) have shown that cases were approximately twelve times more likely (OR = 12: 95% CI: 4-37) to have iron-deficiency anemia compared to controls. In another case-report by Munot and colleagues (306), the authors noted that dietary iron-deficiency was the only common factor among four children suffering from ischemic stroke. Conversely, Roest et al (209) demonstrated that adult female heterozygote carriers for the HFE mutation experienced

an increased risk of cerebrovascular disease (OR = 2.4; 95% CI: 1.3-3.5). Moreover, in an NHANES I follow-up study (n = 5,033), Gillum et al (307) found an increase risk of stroke incidence in white females aged 45-74 with both high and low transferrin saturations. Compared to females with transferrin saturation between 30-36%, those with levels > 44% experienced a RR of 1.96 (95% CI: 1.15-3.36), while those with transferrin saturation < 20% experienced a RR of 1.80 (95% CI: 1.20-2.71). The authors found no such associations in white men aged 45-74 or in African Americans (i.e., male or female).

Iron and chronic obstructive pulmonary disease In the US, COPD is the fourth leading cause of mortality, accounting for 5.3% of total deaths (196). Compared to cardiovascular disease and cancer, less is known about the relationship between iron and chronic obstructive pulmonary disease (COPD). A literature search revealed that lavage samples taken from patients (n = 44) with COPD exhibited elevated concentrations of iron and ferritin compared to healthy controls (n = 50) (308). A recent biomarker study demonstrated the identification of four sensitive and specific biological markers for COPD diagnosis and progression. Three out of four of these markers were iron-specific (i.e., haptoglobin, ceruloplasmin, and hemopexin) (309). The study demonstrated that each of these markers are elevated in patients with COPD, indicative of inflammation. In the presence of infection and inflammation, the iron-regulatory peptide hepcidin is synthesized by the liver. This hormone binds to and degrades the ferroportin receptor on basolateral membrane of enterocytes and outer membrane of macrophages, thereby prohibiting the release of stored iron. In addition, hepcidin sequesters the absorption of dietary iron by enterocytes. Thus, it is reasonable to speculate that patients with COPD may experience higher levels of stored iron compared to their abstaining counterparts.

Supporting this assertion is a recent study of European iron/steel foundry workers (n = 459). In 2011, Bala & Tabaku discovered that iron workers experienced greater than a 2-fold increase in risk of developing COPD compared to non-foundry workers (310). The authors controlled for other germane occupational exposures as well as smoking status. Given the dearth of available evidence, however, it can be concluded that the association between iron and COPD is tentative, at best. More research in this area is required prior to further extrapolation.

Iron and Alzheimer's disease Alzheimer's disease (AD) is the sixth leading cause of mortality in the US, accounting for up to 5.1% of total deaths (196). The relationship between iron and AD has recently been well-studied. Evidence indicates that transferrin receptors (TfRs) are fairly ubiquitous in the human brain (311). Interestingly, TfRs are more highly concentrated in the substania nigra, locus ceruleus, putamen, caudate nucleus, globus pallidus, and hippocampus (311) - regional sites commonly associated with neuronal dysfunction and degradation in Alzheimer's disease (AD) (312-314). Studies have supported this biochemical association by establishing that iron accumulates in regions of the brain of those affected by AD (315-317). In addition, field dependent relaxation rate increase (FDRI) (318, 319) and serial susceptibility weighted (SWI) (318, 320) magnetic resonance imaging (MRI) studies have revealed an age-related increase in iron concentrations in the putamen, caudate, and globus pallidus.

The iron-AD association is further supported by studies demonstrating that iron influences γ-secretase cleavage activity of amyloid precursor protein (APP) (321). Both human (322, 323) and animal studies (324) have confirmed that an increase in labile brain iron coincides with early Aβ plaque formation. Finally, carriers of the HFE C282Y

and H63D variants (mutations in the hereditary hemochromatosis) have been shown in epidemiological studies to display a small increased risk of AD as well as an earlier age of onset of the disease (325-328). However, it should be noted that not all studies examining this association have reported similar findings (329, 330). Similar to persons with high iron stores and abnormal lipid profiles in CVD, significant interactions between the APOEε4 variant and the C282Y and H63D variants have been established in the epidemiological literature for AD as well (331). Heterozygous carriers of both risk variants appear to experience an increased risk of AD compared to heterozygous carriers of either single risk variant alone (331). Thus, the above pathways seemingly converge to implicate a vascular pathway in the development of AD (332). Taken together, the aforementioned results support the hypothesis that iron contributes to the neurodegeneration exhibited in AD via the Fenton Haber-Weiss reaction. The role of iron as an initiator or promoter of this pathway, however, remains to be elucidated.

CHAPTER 4: LITERATURE REVIEW: IRON AND METABOLIC DYSFUNCTION

Epidemiology of Type 2 Diabetes Mellitus (T2DM)

History & Definition Two decades ago T2DM was characterized by a triad, comprised of 1) pancreatic beta cell dysfunction, 2) excess glucose production by the liver and 3) insulin resistance, defined then as an impairment of insulin-mediated glucose uptake into target tissues (e.g., skeletal muscle) (333). This paradigm has more recently been abandoned by most groups in favor of a simpler model where the hallmarks of T2DM are now considered to be elevated fasting insulin concentrations and low insulin secretion by beta cells (334).

Currently, the WHO and IDF define normoglycemia (i.e., normal glucose tolerance (NGT)) as two-hour postprandial (i.e., following a meal) plasma glucose concentrations less than 7 mmol/L (126 mg/dL), impaired glucose tolerance (IGT) as fasting plasma glucose (FPG) concentration less than 7 mmol/L (126 mg/dL) and two-hour postprandial plasma glucose concentrations between 7.8 mmol/L and 11.1 mmol/L (140mg/dL and 200mg/dL), impaired fasting glucose (IFG) as FPG concentrations between 6.1 mmol/L and 6.9 mmol/L (110mg/dL and 125mg/dL) and two-hour postprandial plasma glucose concentrations less than 7.8 mmol/L (140mg/dL) and T2DM as the presence of FPG concentrations greater than or equal to 7 mmol/L (126 mg/dL) or

two-hour plasma glucose concentrations greater than or equal to 11.1 mmol/L (200mg/dL) (335)

Descriptive Epidemiology Given these criteria, the number of individuals affected with T2DM worldwide in 2010 was estimated to be 285 million (333). By the year 2030, this figure is projected to rise to 439 million individuals (336), representing a 54% increase (395). This is not surprising, given the reported 11% conversion rate per year among those with IGT progressing to T2DM, accounting for an increase in the incidence of T2DM of 2 to 4 million new cases per year (333). The risk of T2DM appears to track across increasing levels of FPG concentrations. For example, higher incidence of T2DM is observed among individuals with FPG between 95 to 99 mg/dL compared with those with FPG < 85 mg/dL (HR = 2.33, 95% CI: 1.95-2.79) (337). When classified according to glycated hemoglobin A1C (HbA1c) concentrations, individuals demonstrating the highest risk of developing T2DM are those whose HbA1c level is between 5.7 and 6.4 % (39 to 46 mmol/L) (337).

In 2001, the estimated number of individuals in the U.S. affected with T2DM was 18.1 million, imposing a substantial burden on the healthcare system, with total healthcare costs exceeding $132 billion dollars (333). In 2008, this equated to a mean annual cost per T2DM diagnosis of $881, which was reported to be 21% of the gross domestic product per capita at that time (338).

The National Health Interview Survey (NHIS) and data collected from NHANES have indicated that the prevalence of T2DM doubled from 1990 to 2008 in the U.S. and that there has been no significant change in prevalence from 2008 to 2012 (339). The prevalence of T2DM, however, increases with age from < 1% among those aged 20 years

and younger to 12.3% in those aged 21 to 65 years of age to 25% in those aged 65 years and older (337). Given that one of the largest segments of the population (i.e., Baby Boomers, ages 51-69) is ‘aging-in’ to the group with the highest prevalence of T2DM, the aforementioned stable pattern may soon be disrupted.

Among U.S. adults, the current estimated prevalence of T2DM is reported to range from 11-14%, with males displaying a slight increase in prevalence compared to age-matched females (13.6% vs. 11.2%, respectively) (337). Moreover, the prevalence of T2DM in the U.S. varies across ethnicities, with Non-Hispanic whites demonstrating the lowest prevalence (7.6%), followed by Asian Americans (9%), Hispanic Americans (12.8%) and African Americans (13.2%) (334, 337). The ethnicities with the highest reported prevalence of T2DM are Native Americans and Alaska natives (15.9%) (333).

The same trend appears when examining risks between ethnicities among females, with African American females showing an elevated risk (RR = 1.34, 95%CI: 1.12-1.61) compared to their non-Hispanic white counterparts (340). Hispanic American females show a greater increased risk than both ethnicities (RR = 1.86, 95%CI: 1.40-2.47) (340). Finally, Haffner et al. reported that the observed differences in insulin sensitivity between ethnic groups (i.e., African Americans and non-Hispanic whites) persist after adjustment for BMI, waist-to-hip ratio (WHR), level of physical activity and differences in diet (334). Moreover, the authors concluded that ethnic origin remained a powerful independent predictor of T2DM, accounting for an excess risk of approximately 15% in the Hispanic American population (334). In line with these data, Hispanic Americans demonstrate a higher prevalence of T2DM than do Non-Hispanic whites residing within the same neighborhood (334).

The increased risk of complications among those who eventually progress to T2DM begins years (possibly decades) before diagnosis (341). At diagnosis, approximately 50% of those who have developed T2DM demonstrate overt hypertension or signs of macroangiopathy (341), which increases the risk for subsequent adverse health outcomes/complications such as cardiovascular disease, diabetic neuropathy, nephropathy and retinopathy (336).

Pathophysiology Periods of insulin resistance across the human lifespan are quite common. For instance, puberty, pregnancy and aging are all marked by increases in insulin resistance. However, insulin resistance appears to be a necessary, but not sufficient, condition which must manifest during the progression from normoglycemia to T2DM. Stated otherwise, as long as beta cells exhibit the capacity to compensate for varying degrees of insulin resistance, glucose tolerance remains within normal physiological limits (341).

To develop a complete understanding of the etiology of T2DM, we must first examine the physiology of beta cells as individuals progress across the spectrum from insulin sensitive to insulin resistance. Following a meal, beta cells secrete insulin in a biphasic pattern and the amount of insulin secreted is positively related to systemic glucose concentration. In other words, any acute rise in plasma glucose concentration induces a burst of insulin secretion lasting 5 to 10 minutes (342) and this is referred to as first phase insulin secretion. The first phase typically occurs within 30 minutes of ingesting a meal, serves to prime target (i.e., metabolically active) tissues for forthcoming nutrients and is the main effector which quickly reduces blood glucose levels following a meal (343). The second phase of insulin secretion occurs approximately one- to two-

hours following a meal (i.e., postprandially) and serves to further reduce systemic glycemia.

As glucose tolerance moves from normal to impaired, first phase insulin secretion by beta cells is markedly attenuated (344). This results in an elevated postprandial rise in glycemia over the ensuing two hours. It is postulated that this postprandial rise in glycemia induces beta cells to secrete increasing amounts of insulin following the first phase. Thus, second phase insulin secretion, while less rapid (343), is higher than that of the first phase and functions as a compensatory response (333).

To initially compensate for the perturbance in homeostasis, beta cells begin to hypertrophy, leading to increased beta cell mass (344) and increased insulin output, which results in hyperinsulinemia. The aforementioned compensation does not persist indefinitely, however. As insulin resistance worsens, the pulsatile pattern of first phase of insulin secretion is lost as beta cells begin to undergo apoptosis (333) due to oxidative stress. Eventually, the second phase of insulin secretion begins to decline as apoptosis continues until beta cells no longer secrete insulin (342) and the individual reaches a hyperglycemic state.

Once present, insulin resistance persists (333). However, it appears that it is not a worsening of insulin resistance, per se, which leads to the observed increases in blood glucose concentrations. In other words, a change in the degree of insulin resistance does not appear to account for blood glucose concentrations progressing from NGT to IGT to T2DM (342), as it is common for individuals who develop insulin resistance to never progress to T2DM (344). As mentioned, it appears as if it is the worsening of beta cell compensation that is what is driving the individual across the spectrum from NGT to IGT

to T2DM and that this dysfunction occurs well before blood glucose values mark the presence of IGT (342).

In terms of explaining the etiology and pathogenesis of T2DM, there are two leading hypotheses. One hypothesis states that FFAs and high triglycerides lead to the observed metabolic dysfunction and this is otherwise known as the "lipocentric" view of T2DM (333), which will be discussed in greater detail subsequently. Given that the oxidation of FFAs and glucose both contribute to the pool of metabolic fuel available for energy expenditure, their relative utilization is typically adjusted to match energy supply and demand. It has been postulated that glucose oxidation (i.e., glycolysis) is reduced in favor of FFA oxidation with increasing FFAs within the cytoplasm (345). Moreover, studies have also demonstrated that increased cytoplasmic concentrations of FFAs result in the skewing of the NAD/NADH ratio within the mitochondria, which leads to increased concentrations of acetyl-CoA and increased hepatic glucose production (346). Briefly, during beta oxidation NAD is the oxidizing agent which converts FFAs to acetyl-CoA (346) As the concentration of FFAs increases, the rate of beta oxidation of FFAs to acetyl-CoA within the mitochondria also increases (345, 346). Over time, this is thought to increase the pool of acetyl-CoA with concomitant increases in hepatic glucose output. It must be noted, however, that if the concentration of FFAs does not decrease, the intracellular pool of NAD can become depleted and the NAD/NADH ratio can therefore become skewed, leading to mitochondrial dysfunction. This is thought to lead to the inhibition of FFA beta oxidation, increases in FFA storage as triglycerides, increased glucose output and, as mentioned, mitochondrial dysfunction and apoptosis. Stated differently, given that glycolysis is an energy-conversion pathway, one might hypothesize

that increased FFAs might concomitantly lead to increased intracellular glucose concentrations via competitive inhibition of glycolysis by FFAs as well as increased glucose output via the skewing of the NAD/NADH ratio within the mitochondria. Consistent with this hypothesis, Lam et al. have shown in an animal model that elevation of FFAs is linked to increased hepatic glucose production by increasing gluconeogenesis and this was attributed to an increased intracellular pool of acetyl-CoA (347).

In addition to the above-mentioned metabolic dysregulation, the by-products of excess FFAs (i.e., ceramides) have also been shown to contribute to oxidative stress, which induces beta cell dysfunction and apoptosis (333). Studies which have shown increased triglyceride concentrations in muscle and liver to be a reliable (i.e., consistent) marker of insulin resistance lend further support to this hypothesis (348). Finally, it has been reported that in patients with overt T2DM that the deterioration of insulin sensitivity and secretion due to chronic hyperglycemia (i.e., glucotoxicity) is further aggravated by elevated FFAs (341). Given that elevated FFA concentrations appears to be a consistent feature of subjects with abdominal obesity as well as those diagnosed with T2DM, this hypothesis is plausible.

A second hypothesis proposes that small intracellular amyloid fibrils of islet amyloid polypeptide (IAPP, amylin) leads to beta cell failure during the initial stages of the disease and that T2DM may be a manifestation of beta cell Alzheimer's Disease (349). Amylin is co-secreted with insulin from beta cells and plays a role in glycemic regulation by slowing gastric emptying and promoting satiety (350). The net effect of amylin co-secretion with insulin is therefore to reduce postprandial glucose concentrations. According to this hypothesis, however, co-secretion of amylin is lost,

which leads to the accumulation of the amyloid fibrils through a process that has not been well-characterized. It is thought, however, that the accumulation of amyloid fibrils culminates in mitochondrial dysfunction via interaction with cyclophilin D (CypD), an integral protein of the mitochondrial permeability transition pore (mPTP) (351). Du et al. have shown that the interaction of amyloid fibrils with CypD promotes free radical production as well as the opening of the mPTP, which leads to mitochondrial apoptosis (352).

This hypothesis is consistent with research related to other amyloid diseases, such as Alzheimer's Disease, where the same process is thought to occur. A recent study by Perry et al. lends additional support to this hypothesis. The authors demonstrated that glucagon-like peptide (GLP-1) can modify IAPP processing and protect against oxidative injury (353). Briefly, GLP-1 is an endogenous insulinotropic gut hormone which appears to regulate plasma glucose levels by stimulating insulin secretion via the pancreas in a nutrient-dependent manner (347, 354). The signal transduction pathway of GLP-1 has been elucidated. Briefly, GLP-1 appears to act directly through the cAMP-PKA pathway to enhance and sensitize beta cells to glucose-stimulated insulin secretion (355). Following a meal, GLP-1 stimulates beta cells to increase the rate of glucose metabolism and the production of ATP, which increases the cytoplasmic ATP:ADP ratio. In turn, this leads to depolarization of the plasma membrane via closure of ATP-sensitive potassium channels, thereby permitting the opening of voltage-dependent calcium channels. The influx of cytosolic calcium then triggers the fusion of insulin containing secretory vesicles with the plasma membrane, followed by exocytosis of insulin into circulation.

Interestingly, studies have demonstrated that levels of GLP-1 are reduced among those with T2DM (355), which lends further support to this hypothesis.

Analytic Epidemiology Several studies support the hypothesis that a genetic predisposition for T2DM exists. For example, kinship studies demonstrate an increased (i.e., 2- to 3-fold) risk of developing T2DM among individuals with any first-degree relative diagnosed with the disease (334). Moreover, the risk is amplified (i.e., 5- to 6-fold) among individuals whose parents have been diagnosed with T2DM (334). Investigators agree, however, that any genetic basis for T2DM is complex and that the inherited risk among those with a predisposition for the disease does not follow typical Mendelian patterns (333). In line with this conclusion, candidate gene studies have consistently shown small effect sizes (e.g., 20% - 30%) between single nucleotide polymorphisms (SNPs) in genes involved in beta cell function and T2DM (342, 356). Moreover, effect sizes for SNPs involved in insulin signaling or the glucose transport system are even smaller (356). Thus, environmental factors appear to mediate the development of the disease. For example, the prevalence of diabetes among the Pima Indians living in Mexico is less than 20%, while the risk for T2DM among Pima Indians living in the U.S. is twice that (~ 38%) (337). Given that individuals in each group are very closely related, the observed difference in prevalence rates among these two groups lends support to the role of environmental risk factors.

Apart from a presumed increased risk of T2DM due to genetic predisposition, obesity is among the most highly publicized risk factors for the development of T2DM. Eckel et al. indicate that ~90% of obese patients progress to T2DM (357). Moreover, there appears to be a progressive decrease in insulin sensitivity with increasing degrees of

obesity, especially among those with a family history of T2DM (337). In addition, McColloch et al. have reported that increases in body mass index (BMI) over time appear to be the most important risk factor for progression to T2DM after adjusting for age and race/ethnicity (337). It should be noted here that visceral (i.e., intra-abdominal) fat, as opposed to subcutaneous or retroperitoneal fat, however, appears to confer the greatest risk (333).

Other commonly reported risk factors for the development of T2DM are level of education, socioeconomic status (SES), developed environments, sedentary behavior/level of physical activity, smoking status, consumption of alcoholic beverages, disrupted sleep and diet. Each will briefly be summarized below.

Level of education has consistently been shown to be inversely associated with risk of T2DM across age groups, genders and ethnicities (358-361, 338, 362, 363). In addition, studies have also indicated that there is an inverse relationship between socioeconomic status and the prevalence of T2DM (333, 364). Interestingly, investigations measuring both educational attainment and neighborhood SES indicate that the latter (i.e., low neighborhood SES) appears to confer a greater risk for the development of T2DM, even among those who have high educational achievement (359). In line with these data, Papier et al. have shown that residence in an urban area as a child is associated with the development of T2DM among both males and females (OR = 1.40, 95%CI: 1.10-1.70 and OR = 1.40, 95%CI: 1.01-1.79, respectively), regardless of level of affluence reached as an adult (338).

The association between developed environments and an increased risk of T2DM was highlighted several years ago when populations who rarely experienced T2DM

moved from an agrarian- and/or farming-existence to more developed economies (333). As already mentioned, the prevalence of T2DM among Pima Indians residing in Mexico compared to that of the Pima Indians residing in the southwest U.S. should closely parallel each other if no environmental component were involved in the etiology of T2DM. Moreover, Saharan nomadic tribes and Australian aborigines exhibit dramatic increases in the prevalence of T2DM when moving from an agrarian lifestyle to a developed environment (333).

Physical inactivity (e.g., infrequent exercise, gardening, housework, prolonged television watching and/or computer usage) (338, 334) appears to increase the risk of T2DM (365) and the observed risk persists both in the presence and absence of weight gain (365). For example, low aerobic capacity and muscle strength among a Swedish cohort of males at 18 years of age was shown to be associated with an increased risk of T2DM after a twenty-five-year follow-up period (337). Of note, the same increased risk was noted for males with normal BMI, supporting the hypothesis that lack of physical exertion is a more reliable predictor of T2DM compared to caloric restriction to maintain a given BMI (334). In line with these observations, weight loss via caloric restriction coupled with increased physical activity has been shown to reduce FFAs and triglycerides in adipocytes (347) and reduce the risk of T2DM.

Several studies have shown that current cigarette smoking and excessive alcohol intake increase the risk of T2DM (338). In a meta-analysis of 25 prospective cohort studies, current smokers had a 40% increased risk of developing T2DM (i.e., compared to nonsmokers) (pooled, adjusted RR = 1.40, 95%CI: 1.30-1.60). Moreover, the risk appears to be dose-dependent, with increasing risk with increasing number of cigarettes smoked

per day as well as with pack per year increases (344). Studies examining the effect of smoking cessation have indicated that the risk of developing T2DM reverted to that of never smokers after 20 years of smoking cessation (344)

Sleep apnea, leading to a disrupted sleep cycle, has become a well-established risk factor in the development of T2DM (341, 336). Interestingly, McColloch et al. have shown that both lack of sleep as well as excessive sleep appears to confer sS an increased risk of developing T2DM. Compared with approximately 8 hours a day per night, short duration of sleep (defined as < 5 to 6 hours of sleep per night) and long duration of sleep (defined as > 8 to 9 hours of sleep per night) were significantly associated with an increased risk of T2DM (334). Thus, it appears that the association between sleep and risk of T2DM follows a U-shaped curve. Further research is warranted to understand the impact of sleep patterns on risk of developing T2DM.

The literature indicates that several dietary exposures are associated with either decreasing or with increasing the risk of developing T2DM. Among the exposures which appear to reduce the risk of T2DM are nuts, brown rice, fiber, specific fruits (e.g., blueberries, grapes, apples, bananas, and pears), black coffee, green tea, vitamin D, vitamin E, magnesium and zinc (130, 131). Exposures which are associated with an increased risk of T2DM are sugar intake (e.g., sugar-sweetened soft drinks, sweets), white rice, red meat and processed meat (130, 131) independent of BMI, physical activity, age or family history (334). With regard to red meat and processed meat, iron has consistently been linked to an increased risk of T2DM.

Iron and T2DM The hypothesis that there is an association between iron and T2DM originally derives from studies demonstrating an increased prevalence of T2DM

among individuals with hereditary hemochromatosis (HH) (366), which, as mentioned in chapter 2, manifests due to two missense mutations (C282Y and H63D) in the hemochromatosis gene (*HFE*) located on the short arm (*p*) of chromosome 6 (366). As also mentioned, the *HFE* gene encodes for the HFE protein, which binds to TfR1 and reduces its affinity for transferrin. Mutations of the *HFE* gene, however, result in a loss of this repressor function, which leads to increased iron sequestration in several tissues. Up to 60% of those affected with the condition eventually develop T2DM (Swaminathan), and several authors hypothesize that this is due to an increased accumulation of iron in the pancreas of those who go untreated.

The hypothesis that increased body iron is implicated in the development of T2DM has received experimental support from cell line studies as well as from animal models. For instance, Bothwell et al. reported that the exocrine cells of the pancreas tend to accumulate a greater portion of the metal, yet remain functional (367). Interestingly, the authors report that while beta cells acquire less iron than exocrine cells, they appear to be more sensitive to the metal, which is most likely due to their low expression of antioxidants (e.g., catalase and superoxide dismutase 2 (SOD2)) (367). Ultimately, this sensitivity is thought to manifest as an impairment of insulin synthesis (368). In a mouse model of HH, iron excess and oxidative stress were shown to meditate apoptosis of pancreatic islets with a resultant decrease in insulin secretory capacity (369). Finally, several epidemiological investigations, including 10 cohort studies, 8 case-control studies and 8 cross-sectional studies have reported associations between iron stores and T2DM. Each study will be briefly summarized below.

During a 12-year prospective cohort study using data obtained from 33,541 participants in the Health Professionals' Follow-up Study, Jiang et al. grouped participants into quintiles of increasing intake of heme iron and heme iron from red meat, respectively. Compared to the lowest quintile of heme iron intake, participants in the highest quintile of heme iron intake had a RR of 1.28 (95% CI: 1.02-1.61, $P_{trend} = 0.05$) for developing T2DM. Moreover, participants in the highest quintile of heme iron from red meat (compared to the lowest quintile of heme iron from red meat) had a RR of 1.63 (95% CI: 1.26-2.10, $P_{trend} < 0.001$) for developing T2DM (1).

In another prospective nested case-control study (n = 698 cases, n = 716 controls) matched on age, race, fasting status and BMI, Jiang et al. followed 32,826 females participating in the Nurses' Health Study over 10 years from 1989 through 1990. The authors observed a higher incidence of T2DM among cases as well as higher serum ferritin among cases compared to controls (109 ± 105 ng/mL (cases) vs 71.5 ± 69 ng/mL (controls), $P < 0.001$). Moreover, the authors reported that the risk of incident T2DM increased across increasing quintiles of ferritin among cases (RR_{Q2} = 1.09 (95%CI: 0.70-1.70), RR_{Q3} = 1.26 (95%CI: 0.82-1.95), RR_{Q4} = 1.30 (95%CI: 0.83-2.04) and RR_{Q5} = 2.68 (95%CI: 1.75-4.11, $P_{trend} < 0.001$). The associations persisted after adjustment for menopausal status, alcohol consumption and C-reactive protein (370).

Lee et al. followed 35,698 postmenopausal females aged 55 to 69 years for 11 years to examine the effect of dietary exposures on incident T2DM. Over the follow-up period, baseline heme iron intake was associated with incident T2DM across increasing quintiles of intake (RR_{Q2} = 1.07 (95%CI not provided), RR_{Q3} = 1.12 (95%CI not provided), RR_{Q4} = 1.14 (95%CI not provided) and RR_{Q5} = 1.28 (95%CI: 1.04-1.58), P_{trend}

= 0.02) after adjustment for non-dietary and dietary risk factors. The authors were also able to show an interaction between heme iron intake, alcohol consumption and T2DM. For example, the association between heme iron intake and T2DM was marginal among non-drinkers, but grew in magnitude among participants who consumed greater quantities of alcohol. In a model restricted to those who consumed at least 15 grams of alcohol per day, the RR across increasing quintiles of heme iron intake were RR_{Q2} = 2.26 (95%CI: 0.79-6.44), RR_{Q3} = 3.22 (95%CI: 1.16-9.84), RR_{Q4} = 1.92 (95%CI: 0.63-5.87) and RR_{Q5} = 4.42 (95%CI: 1.37-14.25), P_{trend} = 0.05 (371).

In 2004, Song et al. followed 37,309 participants aged 45 years and older in the Women's Health Study for an average of 8.8 years who were free from cardiovascular disease, cancer, and T2DM at baseline to examine the association between red and processed meat intake and incident T2DM. During 326,876 person-years of follow-up, the authors reported positive associations between intakes of red meat and processed meat and risk of type 2 diabetes after adjustment for age, BMI, total energy intake, exercise, alcohol intake, cigarette smoking, and family history of diabetes. Compared to the females in the lowest quintile of red meat intake to females in the highest quintile of red meat intake, the RR for developing T2DM was 1.28 (95%CI: 1.07-1.53, $P_{trend} <$ 0.001). Moreover, the RR for developing T2DM was 1.23 (95%CI: 1.05-1.45, P_{trend} = 0.001) among females in the highest quintile of processed meat intake compared to those in the lowest quintile of processed meat intake. The authors also reported an increased risk of developing T2DM among females who more frequently consumed processed meat, defined as consuming processed meat ≥ 5 days per week compared to consumption

less than once a month (RR 1.43, 95%CI: 1.17-1.75, $P < 0.001$) after adjustment for dietary fiber intake, magnesium, glycemic load and total fat intake (372).

Fumeron et al. randomly selected 1,277 (i.e., 644 males and 633 females) participants from a pool of 4,501 individuals in the DESIR cohort to examine parameters of iron status at baseline with subsequent development of T2DM over a 3-year follow up period. The authors reported that baseline serum ferritin concentrations were positively correlated with fasting plasma insulin as well as fasting plasma glucose. Moreover, baseline serum ferritin concentration was reported to be an independent predictor of increases in plasma insulin concentrations over the 3-year follow-up period ($P = 0.002$). Finally, the authors noted that baseline serum ferritin and transferrin were independently associated with the onset of hyperglycemia over the 3-year follow-up period, regardless of gender ($P < 0.001$) (373).

Rajpathak et al. conducted a prospective cohort study within the Nurses' Health Study, following 85,031 healthy females aged 34-59 years from 1980 to 2000 to examine the association between total iron intake, total dietary iron intake, supplemental iron intake and nonheme iron intake and the development of T2DM. Following the 20-year follow-up period, the authors reported no associations between total, dietary, supplemental or nonheme iron and the risk of T2DM. However, the authors did observe positive associations across increasing quintiles of heme iron intake and the risk of T2DM: $RR_{Q2} = 1.08$ (95%CI: 0.97-1.19), $RR_{Q3} = 1.20$ (95%CI: 1.09-1.33), $RR_{Q4} = 1.27$ (95%CI: 1.14-1.41) and $RR_{Q5} = 1.28$ (95%CI: 1.14-1.45, $P_{trend} < 0.0001$) after adjustment for age, BMI and other nondietary and dietary risk factors (374).

Using data obtained from 360 T2DM cases and 758 controls nested within the EPIC study, Fourouri et al. sought to examine the association between sex-specific baseline quintiles of serum ferritin concentration and T2DM. The authors reported that the baseline serum ferritin geometric mean was higher among cases than control participants in both males and females (males: 96.6 ng/mL (cases) vs 67.8 ng/mL (controls), $P < 0.001$; females 45.9 ng/mL (cases) vs 34.8 ng/ml (controls), $P = 0.005$). Moreover, the risk of incident T2DM was higher among those in the highest quartile of serum ferritin compared to those in the lowest quartile (OR = 7.4, 95%CI: 3.5-15.4, $P < 0.0001$) after adjustment for age, BMI, sex, family history, physical activity, smoking, C-reactive protein, IL-6 and fibrinogen (375).

In 2007, Jehn et al. conducted a case-cohort study that was nested within the Atherosclerosis Risk in Communities (ARIC) Study (n = 599 cases, n = 690 controls obtained from a random sample of the cohort) to examine the association between serum ferritin and risk of T2DM after an 8-year follow-up period. Compared to cases in the lowest quintile of serum ferritin, cases in the highest quintile of serum ferritin had an increased risk of developing T2DM (HR = 1.74, 95%CI: 1.14-2.65, $P_{trend} < 0.001$) after adjustment for age, gender, menopausal status, ethnicity, center, smoking status and alcohol intake (377).

In a study published in 2008, Le et al. reported results from prospective cohort data from 5,512 male and female participants in the Aerobics Center Longitudinal Study (ACLS) between 1995 and 2001. Over the 5-year follow-up period, the authors reported that serum ferritin concentrations were significantly higher in males than in females (148.5 ± 104.7 ng/ml vs. 52.2 ± 45.9 ng/ml) and that male participants in the highest

quartile of serum ferritin had a greater risk of developing T2DM compared to those in the lowest quartile of serum ferritin (HR: 1.67, 95%CI: 1.05- 2.66, $P = 0.027$). While a similar trend was observed for females, the comparisons across quartiles of serum ferritin were not statistically significant (377).

In contrast to the above positive associations between iron and the development of T2DM, in 2002 Mainous et al. reported that elevated serum transferrin saturation was not significantly associated with the development of T2DM in participants in NHANES. For example, the authors reported that the incidence of T2DM was 7.5% among individuals with serum transferrin saturation > 55% and 10.2% among individuals with serum transferrin saturation ≤ 45% ($P = 0.38$) and concluded that increased body iron was not associated with an increased risk of T2DM (378). It must be noted, however, that while Mainous et al. have published several studies using NHANES data, it is not clear as to how the authors accounted for the complex study design or sampling strategy.

In 1988, Jones et al. were among the first groups to publish a case-control study evaluating the association between indices of body iron and T2DM (n = 25 cases, n = 37 controls). The authors reported that serum ferritin concentration was increased in both males (124 ± 113 ng/mL (cases) vs 44 ± 38 ng/mL (controls), $P < 0.001$) and females (132 ± 118 ng/mL (cases) vs 25 ± 12 ng/mL (controls), $P < 0.001$) (379).

Several years later in 2006, Adou-Shousha aimed to investigate the correlation between serum ferritin concentrations, sTfR and IL-8 concentrations among 20 females with T2DM who were matched on age to 10 healthy controls. The authors reported that serum ferritin was positively associated with IL-8 concentrations in those with T2DM ($P = 0.0029$) and not in controls. A similar positive association was observed between sTfR

and IL-8 concentrations across groups (*P* = 0.032) (380). Taken together, these results support the association between increased iron and T2DM.

In 2008, Smotra conducted a case-control study (n = 50 cases, n = 50 controls) in a tertiary care hospital in North India to examine the relationship between serum ferritin concentrations and serum insulin concentrations among those with and without T2DM. The authors matched cases to controls on age, sex and BMI and reported a positive correlation between serum ferritin and serum insulin concentrations (r = 0.77, *P* = 0.01) and this corresponded to poor glycemic control (i.e., higher HbA1c concentrations) among those with T2DM (381)

Waheed (2009) published a small case-control study (n = 30 cases, n = 30 controls) using data collected from diabetic clinics of Rawalpindi in Pakistan. The authors reported that cases had higher fasting glucose concentrations, HbA1c, total cholesterol, LDL cholesterol, triglycerides and ferritin compared to controls (*P* < 0.001), while HDL cholesterol was lower in those with T2DM (*P* < 0.001) (382).

Using controls obtained from two population-based studies (i.e., Copenhagen City Heart Study (CCHS, N = 9,121) and The Copenhagen General Population Study (CGPS, N = 24,195), Ellervik et al. 5:1 matched controls to 6,129 T2DM cases from the Steno Diabetes Centre on age and sex. Compared to cases with transferrin saturation < 50%, cases with transferrin saturation ≥50% had increased odds of developing T2DM: OR = 1.7 (95%CI: 1.4-2.1; *P* = 0.001) (383).

In contrast to the above positive associations between indices of body iron and prevalence of T2DM, some authors have reported no associations between indices of body iron and T2DM (i.e., the associations were not statistically significant). For

instance, Skomro et al. consecutively enrolled 58 T2DM cases and 48 controls into a sleep study in 2001, seeking to examine the association between sleep complaints and T2DM. However, the authors also collected data on biochemical parameters (including creatinine, urea, folate, vitamin B12 and serum ferritin) and reported that cases had higher serum ferritin compared to controls (154.7 ± 178.6 µg/L (cases) vs 107 ± 95 (controls)) but this was not statistically significant ($P = 0.14$). While not statistically significant, the results appear support the aforementioned association between increased body iron and T2DM (384)

A few years later, Elis et al. sought to evaluate the relationship between diabetic retinopathy and serum ferritin levels in a sample of T2DM cases and controls. The study sample was comprised of 3 groups: 1) individuals with T2DM and severe diabetic retinopathy (n = 22), 2) individuals with T2DM without retinopathy (n = 29) and 3) individual controls free from disease (n = 40). The groups were matched on age, gender and hemoglobin levels and comparisons between serum iron, transferrin and serum ferritin concentration were made across each of the three groups. While the authors reported higher concentrations of serum ferritin in the T2DM groups, the comparisons were not statistically significant compared to controls ($P = 0.21$). Moreover, the authors reported that there was no apparent correlation between serum iron or serum ferritin and HbA1c concentration between the groups comprised of individuals with T2DM. Thus, the authors concluded that iron does not appear to have a major role in the development of T2DM or diabetic retinopathy (385). Given the overall small sample size (n = 91), which was stratified into groups of 3, it appears that one cannot draw any valid conclusions based upon these data.

Finally, Rajpathak et al. conducted a nested case-control study (280 cases and 280 age and sex matched controls) within the placebo arm of the Diabetes Prevention Program and reported that cases had higher sTfR levels compared to controls (3.50 ± 0.07 mg/L vs. 3.30 ± 0.06 mg/L, $P = 0.03$) and that serum ferritin levels did not differ statistically between cases and controls. Cases in the highest quintile of sTfR concentrations were observed to have an increased risk of developing T2DM compared to those in the lowest quintile of sTfR concentrations (OR = 2.26 (95%CI: 1.37-4.01, $P = 0.008$) (386). The results of this study allude to an increased requirement for iron as opposed to an increased storage of iron being associated with T2DM, which could be an artefact of the tendency of iron as a pro-oxidant to promote disease progression.

Among the cross-sectional studies reporting associations between body iron and T2DM, Hughes et al. in 1998 were one of the first groups to show that serum ferritin concentrations were higher in individuals with T2DM than those unaffected with the condition. Using data obtained from participants in The National University of Singapore Heart Study (i.e., a cross-sectional study comprised of a random sample of individuals aged 30-69 years) the authors observed mean serum ferritin concentrations of 231 µg/L (range: 205-257 µg/L) in males affected with T2DM (n = 72), compared to 181 µg/L (range: 167-195 µg/L) in males who were unaffected (n = 248, $P < 0.01$). Moreover, the same trend was observed among females, where those affected with T2DM (n = 54) had higher mean serum ferritin concentrations than unaffected females (n = 282) (128 µg/L (range: 98-150 µg/L) vs 77 µg/L (range: 64-90 µg/L, $P < 0.01$)) after adjustment for age and ethnicity (387).

A year later Cogswell et al. examined the association between serum ferritin concentration, glucose tolerance status, insulin concentration, glucose concentration and glycosylated hemoglobin among 9,486 U.S. adults aged 20 years using data obtained from NHANES III (1988–1994). The authors created a dichotomous categorical variable from ferritin concentrations (i.e., < 300 µg/L vs. ≥ 300 µg/L in males and < 150 µg/L vs. ≥ 150 µg/L in females) and reported an increased risk for T2DM for both males and females using these cutoffs (OR = 4.94 (95%CI: 3.05–8.01) for males and 3.61 (95%CI: 2.01–6.48) for females) after adjustment for age, sex, ethnicity, education, BMI, alcohol consumption, alanine aminotransferase concentration, C-reactive protein concentration, and examination session attended (388).

In 2006, Shi published the results from a cross-sectional household survey administered to 2,849 males and females aged 20 years and older in the Jiangsu Province of China and reported that both mean hemoglobin and serum ferritin concentrations increased with increasing FPG. Moreover, the authors observed an increased risk of T2DM for females in the highest quartile of hemoglobin concentration as well as those in the highest quartile of serum ferritin concentration compared to females in the lowest quartiles of hemoglobin and ferritin concentration, respectively (OR = 2.15 (95%CI: 1.03-4.51), hemoglobin; OR = 3.79 (95%CI: 1.72-8.36, serum ferritin). In addition, females in the highest quartile of dietary iron intake had an increased risk of T2DM compared to females in the lowest quartile of dietary iron intake (OR = 5.53 (95%CI: 1.47-20.44). Similar trends were observed in males. However, none of the comparisons across quartiles were statistically significant (389)

In another cross-sectional study conducted a few years later, Kim et al. (2008) analyzed data obtained from 6,346 nonpregnant females participating in NHANES III (1988–1994). The authors categorized females into three groups: 1) those who self-reported a history of gestational diabetes (n = 87), 2) those with diagnosed T2DM (n = 244), or 3) those with neither condition (i.e., controls) (n = 6,015) and observed that serum ferritin was higher in females with T2DM compared to controls (120.3 ng/mL vs 66.3 ng/mL, $P < 0.05$) (390).

Luan (2008) examined the association between self-reported dietary iron intake (i.e., total iron intake and nonheme iron intake), serum ferritin and T2DM among 2,997 individuals aged ≥ 18 years using data obtained from household surveys administered in the Liaoning Province in northern China. The authors reported that serum ferritin was positively associated with the risk of T2DM after adjustment for age, sex, non-dietary exposures and dietary exposures. No associations were observed between total iron intake, nonheme iron intake and risk of T2DM. However, higher self-reported heme iron intakes were significantly associated with an increased risk of T2DM after adjustment for age, sex, nondietary factors and dietary exposures (391).

Sun (2008) et al. sought to examine the association between serum ferritin concentrations and T2DM in a sample of 3,289 participants aged 50-70 years of age from Beijing and Hanghai China during 2005. Not surprisingly, the authors noted that median ferritin concentrations were higher in males compared to females (155.7 ng/mL in males and 111.9 ng/mL in females). In addition, the authors reported an increased risk of T2DM among participants in the highest quartile of serum ferritin concentration compared to those in the lowest quartile of serum ferritin concentration (OR = 3.26, (95%CI: 2.36-

4.51)) after adjustment for other dietary exposures, BMI, inflammatory markers, and adipokines (392).

A few years later, Kim et al. analyzed clinical and laboratory data obtained from 6,378 males and 5,712 females aged 20-89 years undergoing general medical checkups at the Health Promotion Center at Asan Medical Center in Seoul, Korea. The study sample included 1,054 subjects diagnosed with T2DM, 3,783 subjects with IFG and 7,253 subjects with NGT. The authors observed that serum ferritin concentrations were the highest those with T2DM, followed by those in the IFG group and the NGT group, regardless of gender (186 ± 127 ng/mL (T2DM), 176 ± 108 ng/mL (IFG), and 156 ± 92 ng/mL (NGT), respectively, in males; 85 ± 62 ng/mL (T2DM), 75 ± 55 ng/mL (IFG), and 59 ± 47 ng/mL (NGT), respectively, in females)). In addition, the authors reported an increased risk for T2DM among those in the highest quartile of serum ferritin concentrations compared to those in the lowest quartile of serum ferritin concentrations (OR = 1.71 (95%CI: 1.38-2.12) in males and OR = 1.50 (95%CI: 1.05-2.13) in females) (393).

Finally, Lee et al. (2011) examined the association between serum ferritin concentrations and T2DM among 6,311 individuals aged 20 years and older using data obtained from the 2008 Korean National Health and Nutrition Examination Survey. The authors reported that T2DM was more prevalent among individuals in the highest quartile of serum ferritin concentrations compared to those in the lowest quartile of serum ferritin concentrations in males (n = 2,684) (prevalence = 5.27% (95%CI: 3.13-7.41) vs. 1.42 (95%CI: 0.40-2.44, $P < 0.001$), pre-menopausal females (n = 2,120) (prevalence = 7.34% (95%CI: 6.28-8.40) vs. 1.95 (95%CI: 0.95-2.95, $P < 0.001$), and post-menopausal

females (n = 1,507) (prevalence = 15.97% (95%CI: 10.91-21.02) vs. 8.68 (95%CI: 5.01-12.34, $P < 0.001$) (394)

More recently, two groups have each conducted separate meta-analyses examining the association between iron parameters (i.e., serum ferritin, heme iron intake) and T2DM with the above-mentioned studies. In 2013, Orban et al. included a total of 13 studies in the calculation of pooled effect sizes for the association between the highest versus the lowest categories of serum ferritin concentration and risk of T2DM (Table 8). Overall, the pooled effect size across studies comparing the highest versus the lowest category of serum ferritin among study participants with T2DM was 1.67 (95%CI: 1.41-1.99, $P < 0.001$, I-squared = 61%). A strength of the meta-analysis was that the authors were able to conduct subgroup analyses among studies adjusting for inflammation (e.g., C-reactive protein (CRP)) and reported a pooled effect size among studies controlling for CRP of 1.61 (95%CI: 1.18-2.20, $P = 0.002$) compared to 1.67 (95%CI: 1.23-2.27, $P = 0.001$) for studies not controlling for CRP (395).

In 2012, Zhao et al. published a meta-analysis which included a subset of the aforementioned studies (N = 12) which excluded a report that did not collect data on heme and non-heme iron intake (Table 8), and reported a pooled effect size of 1.31 (95%CI: 1.20-1.43, I-squared = 0%) comparing individuals in the highest category of heme iron intake to those in the lowest category of heme iron intake. Moreover, when the authors stratified their analyses by study design (i.e., cohort vs cross-sectional) using the same studies as Oraban et al., they reported a similar pooled effect size for the association between serum ferritin and T2DM among cohort studies: RR = 1.66 (95%CI: 1.15-2.39, I-squared = 66.3%). The pooled effect size for the association between serum ferritin and

T2DM among cross-sectional studies was higher (RR = 2.29 (95%CI: 1.48-3.54, I-squared = 88.7%)) (396). These results indicate that the pooled effect sizes obtained by Orban et al. may not be heavily influenced by study design, given that the pooled effect size for cohort studies calculated by Zhao et al. were of the same magnitude of as those calculated by Orban et al., who did not stratify effect size calculation by study design.

Interventional Epidemiology Additional support for a positive association between body iron and T2DM has been obtained interventional studies among HH patients whose body iron stores were reduced with phlebotomy and/or iron chelation therapy (397, 398). These investigators report that HH patients experienced improvements in glycemic control and 30-40% of patients in the study conducted by Facchini et al. were able to eliminate oral hypoglycemic therapy or were able to decrease their current dosage (398).

Conclusion Taken together, it appears as though iron stores are positively associated with the development of T2DM. It should be noted, however, that each of the above studies used serum ferritin and/or transferrin saturation as indices of iron stores. As already mentioned in Chapter 1, these parameters demonstrate diurnal variation and/or can be influenced by inflammation. Therefore, predictive, non-invasive, and inexpensive analytes unaltered by inflammation would be valuable for use in monitoring the iron status of those at risk for developing T2DM. To my knowledge, no study to date has employed the algorithm developed by JD Cook et al. in 2003 (50) as an index for iron repletion when examining the association between iron stores and T2DM. As mentioned, the index is a mathematically-derived ratio of soluble transferrin receptor and ferritin

found in serum and appears to be a more stable index of iron stores than either analyte used independently (51-52).

Epidemiology of metabolic syndrome

History & Definition The modern concept of what is referred to as metabolic syndrome originated with Gerald Reaven in 1988 (399). Reaven postulated that insulin resistance was the driver of glucose intolerance, hyperinsulinaemia, increased very-low-density lipoproteins (VLDLs), decreased high-density lipoproteins (HDLs) and hypertension (400.). Twenty years and a few different naming conventions later (e.g., Reaven's syndrome, Syndrome X, CHAOS (i.e., coronary artery disease, hypertension, atherosclerosis, obesity and stroke), insulin resistance syndrome) the aforementioned cluster of metabolic abnormalities has become known as the metabolic syndrome (400, 401). The benefit of utilizing the term metabolic syndrome is not so much in identifying patients with a general risk of cardiovascular disease and T2DM, but more so with the identification of specific subgroups of individuals with a shared pathophysiology (402). It should be noted here that the clinical utility of the term, however, has been criticized. It has been suggested that the 'syndrome' may not confer any greater risk than that explained independently by each of its components. Thus, it appears that the term metabolic syndrome should not be misused to refer to a disease unto itself (403), as there is a lack of treatment options available to ameliorate the cluster beyond those already employed to treat each individual component (404).

The most frequently used definitions to determine the presence of metabolic syndrome include the National Cholesterol Education Program Adult Treatment Panel III

(NCEP-ATP III), criteria published in 2001 and updated in 2005, the International Diabetes Federation Criteria (IDF) proposed in 2004 and the WHO definition published in 1999 (405). The WHO definition requires that T2DM or impaired fasting glucose first be present in addition to any two of the following: obesity, dyslipidemia (i.e., increased triglycerides or decreased HDL), hypertension or microalbuminuria. The IDF criteria requires that obesity first be present in addition to any two of the following: impaired fasting glucose, increased triglycerides, decreased HDL or hypertension. The NCEP-ATP III criteria takes into account the same components as the IDF criteria. However, the NCEP-ATP III criteria treats all components equally and specifies that if at least three of the components are manifest, then metabolic syndrome is present. In 2009, several organizations (i.e., the IDF, the National Heart, Lung, and Blood Institute of the U.S., the American Heart Association, the World Heart Federation, the International Atherosclerosis Society and the International Association for the Study of Obesity) issued a joint statement on the definition of metabolic syndrome which included criteria upon which they have all agreed. These criteria are thus referred to as the "harmonized" criteria for metabolic syndrome. The updated criteria contained in the joint statement is essentially the NCEP-ATP III criteria with population- and country- specific definitions for obesity (i.e., waist circumference) (406.).

Descriptive Epidemiology Applying the NCEP-ATP III definition for metabolic syndrome, the age-adjusted prevalence in the U.S. using data obtained from 8,814 participants in NHANES III has been estimated to be 23.7% (24.0% in males, 23.4% in females) (407, 408). However, more recent prevalence estimates of metabolic syndrome among U.S. adults demonstrate an increase in the prevalence of metabolic syndrome in

both males (30.3%) and females (35.6%), suggesting that the prevalence of metabolic syndrome in females has surpassed that among males (409). In line with these data, Ramos and Olden reported that approximately 50% (n = 643) of their study population of U.S. females of childbearing age either had metabolic syndrome or were at an increased risk for developing it (410). Based upon these estimates, it appears that metabolic syndrome in U.S. females is a growing public health concern.

The prevalence of metabolic syndrome increases with age from 6.7% among those aged 20-29 years to 44% in those aged 60-69 years (408) to over 50% in those aged 70 years and older (411). Given that the prevalence of each component of metabolic syndrome (i.e., obesity, hypertension, dyslipidemia and hyperglycemia) has also been observed to increase with age, it is not surprising that increasing age appears to be a determinant in the prevalence of metabolic syndrome.

The prevalence of metabolic syndrome has also been observed to differ across ethnicities. For example, Hispanic Americans having a disproportionately higher prevalence of metabolic syndrome compared to other ethnicities (35.4%), followed by non-Hispanic whites (33.4%) and African Americans (32.7%) (409). This pattern holds when stratified by male gender and ethnicity, where the highest prevalence is found among Hispanic American males (37%), followed by non-Hispanic white males (25%) and the lowest prevalence is found among African American males (21%) (407, 411). The prevalence of metabolic syndrome among adult U.S. females is the highest among Hispanic American females (41%), followed by African American females (26%) and the lowest in non-Hispanic white females (21%) (407). The observed differences in the prevalence of metabolic syndrome across ethnicities has been postulated to be driven by

disparities in access to healthcare (412, 413), level of education (412), income (412) and level of urbanization (414).

Pathophysiology Although the etiology of metabolic syndrome is uncertain, several hypotheses implicate insulin resistance, visceral adiposity and low-grade inflammation as the drivers of each downstream component (400). As mentioned previously, insulin resistance has been defined as the inability of insulin to stimulate adequate glucose uptake in muscle and adipose tissue as well as the inability of insulin to suppress hepatic glucose output (415). Visceral adiposity (i.e., central obesity) commonly refers to increased visceral adipose tissue within the peritoneal cavity and inflammation in metabolic syndrome is defined by the presence of inflammatory cytokines/adipokines. While adipose tissue is an important source of chronic inflammation, it should be noted that other cell types within adipose tissue (e.g., macrophages) contribute substantially to the amount of inflammation present (405).

Among these three factors, visceral adiposity appears to be the most salient in the etiology of metabolic syndrome (416), as visceral adipose tissue releases FFAs directly into portal circulation (416). Moreover, the literature indicates that FFAs accumulate in the liver, pancreas, heart and other organs with increasing adiposity (415). Eventually, this culminates in organ dysfunction, resulting in impaired insulin, glucose and cholesterol regulation, which is commonly referred to as “lipotoxicity.” In addition, there is a significant infiltration of macrophages in adipose tissue with increasing adiposity (415) and it has been shown that adipose tissue secretes monocyte chemotactic protein-1 (MCP-1), which mediates macrophage recruitment to sites of inflammation. Briefly, MCP-1 is a chemokine whose production is primarily stimulated by inflammation and

oxidative stress, regulates migration and infiltration of reticuloendothelial cells (i.e., monocytes and macrophages) to tissues secreting the protein.

Levels of systemic and adipose tissue MCP-1 are elevated in rodent models of obesity and targeted ablation of MCP-1 or its receptor reduces adipose tissue macrophage infiltration (417). This has been shown to improve insulin sensitivity. Conversely, insulin resistance and increased infiltration of macrophages within adipose tissue have been observed in models of MCP-1 overexpression (405). Finally, recruitment of macrophages via adipocyte secretion of MCP-1 leads to their subsequent activation and secretion of TNF-alpha and IL-6.

In terms of other metabolic syndrome components, dyslipidemia appears to be secondary to increased visceral adiposity and the development of insulin resistance due to the effect of adipose tissue secretion of FFAs. In muscle, FFAs increase insulin resistance by inhibiting insulin-mediated glucose uptake via decreasing GLUT4 expression (418). The ensuing increase in circulating glucose thus induces beta cell insulin secretion, resulting in hyperinsulinemia. Moreover, FFAs directly increase the production of glucose, triglycerides and the secretion of VLDLs in the liver. For example, FFAs reduce the conversion of glucose to its storage form (i.e., glycogen) which leads to subsequent hyperglycemia with a concomitant increase in lipid accumulation in the form of triglycerides. The ensuing hypertriglyceridemia is the result of both an increase in VLDL production as well as a decrease in VLDL clearance. In other words, triglycerides stored in VLDLs are transferred to HDL by the cholesterol ester transport protein (CETP) in exchange for cholesteryl esters, resulting in triglyceride-enriched HDL and cholesteryl ester-enriched VLDL particles (419). It has been shown that the triglyceride-enriched

HDL particle is a more specific substrate for hepatic lipase (420). Thus, HDL is cleared more rapidly and VLDLs less rapidly from circulation, resulting in fewer HDL particles to participate in reverse cholesterol transport from the vasculature (419).

Endothelial dysfunction (i.e., hypertension) also appears secondary to increased visceral adiposity and the development of insulin resistance, most likely due to the increase in FFAs that are released from visceral adipose tissue. In line with this hypothesis, 15% of normal-weight individuals in the U.S. have hypertension, whereas the prevalence of hypertension among obese individuals is 42% (421). Moreover, data from the Framingham Heart Study indicates that ~80% of essential hypertension in males and 65% in females can be directly attributed to obesity (422). While the relationship between obesity, insulin resistance and hypertension has been well established, several different mechanisms have been proposed.

One hypothesis is that FFAs are pressors and can themselves mediate vasoconstriction, as FFAs have been shown to increase angiotensinogen production by vascular smooth muscle cells (VSMCs) in culture (423). In line with these data, Egan et al. have also shown that increased adipocyte mass (i.e., hypertrophy) leads to increased angiotensinogen production in adipocytes (424). While the liver is the primary site of angiotensinogen production, it appears as though it is not the only site, and production of angiotensinogen in other tissues is thought to be affected by increasing concentration of FFAs (423).

Another hypothesis involves insulin and nitric oxide (NO). Endothelin-1, a potent vasoconstrictor, is activated by insulin through the MAP kinase (MAPK) pathway (425). Thus, increasing concentrations of insulin within circulation appears to have a

vasocontricting effect (421, 426). Under normal physiologic conditions, NO inhibits the secretion of endothelin-1 (415). However, NO production decreases with increasing adiposity (427). Thus, in a hyperinsulinemic state the balance between endothelin-1 and NO is lost (421).

Analytic Epidemiology When considering risk factors associated with the development of metabolic syndrome, one must consider the risk factors associated with each component (i.e., obesity, hypertension, dyslipidemia and hyperglycemia) of metabolic syndrome. The literature indicates that the obesity component of metabolic syndrome (i.e., defined using WHO criteria as BMI > 29.9) is the single most important risk factor for the development of metabolic syndrome. Contributing to this trend is the fact that most parts of the world have moved from an era of persistent famine to one of nutrient excess comprised of high-fat and high-carbohydrate diets, concomitant decreases in physical activity and increased smoking and ethanol use.

With respect to other components of metabolic syndrome, the risk factors implicated in the development of hypertension and hyperlipidemia also appear to overlap with those of the obesity component (e.g., unhealthy diet which is rich in sodium, lack of physical activity, smoking, alcohol use). Finally, while the risk factors for the development of hyperglycemia overlap somewhat with those of obesity, it should be noted that increased intake of sweets (e.g., sugar-sweetened beverages, candy) are additional risk factors associated with development of the hyperglycemia component of metabolic syndrome. Finally, several studies indicate that intake of red meat and indices of iron status are independently associated with an increased risk of metabolic syndrome.

Conclusion The development of metabolic syndrome has been shown to increase the probability of progressing to T2DM up to fourfold (428) and the risk of progression increases with increasing number of metabolic syndrome components (429). Given that the prevalence of two or more metabolic syndrome components in the U.S. is 43.9%, public health officials are interested in determinants of the condition which are modifiable through primary prevention efforts. While several authors postulate that conversion from metabolic syndrome to T2DM appears to be primarily driven by the hyperglycemia component of metabolic syndrome, other groups postulate that dietary iron is a modifiable exposure that is directly involved in the initiation, promotion and progression of the condition (430).

Iron and metabolic syndrome Similar to T2DM, the hypothesis that there is an association between iron and metabolic syndrome derives from studies demonstrating an increased prevalence of metabolic syndrome among individuals with HH (366). Several epidemiological investigations, including 2 cohort studies, 2 case-control studies and 13 cross-sectional studies have reported associations between iron stores and metabolic syndrome. Each study will be briefly summarized below.

In 2007 Vari et al. followed a total of 944 individuals 30-65 years of age who were enrolled in the DESIR cohort (i.e., 469 males and 278 premenopausal and 197 postmenopausal females) for a period of 6 years. At the end of the follow-up period, the authors reported increased odds of developing metabolic syndrome among those in the highest tertile of log(ferritin) concentrations compared to those in the lowest tertile of log(ferritin) concentrations: OR = 1.49 (95%CI: 1.14-1.94) for males, OR = 2.10 (95%CI: 1.27-3.48) for premenopausal females and OR = 1.80 (95%CI: 1.21-2.68) for

postmenopausal females. In addition, the authors reported increased odds of developing metabolic syndrome among those in the highest tertile of transferrin concentrations compared to those in the lowest tertile of transferrin concentrations: OR = 1.94 (95%CI: 1.53-2.47) for males, OR = 2.22 (95%CI: 1.32-3.75) for premenopausal females and OR = 2.14 (95%CI: 1.47-3.10) for postmenopausal females. At the end of the follow-up period, the authors observed that incident metabolic syndrome was associated with higher serum ferritin and transferrin at baseline across all three groups: OR = 1.46 (95%CI: 1.13-1.89) for males, OR = 1.28 (95%CI: 0.85-1.94) for premenopausal females and OR = 1.62 (95%CI: 1.10-2.38) for postmenopausal females for serum ferritin. For transferrin, the OR = 1.41 (95%CI: 1.10-1.81) for males, OR = 1.63 (95%CI: 1.05-2.52) for premenopausal females and OR = 1.51 (95%CI: 1.02-2.22) for postmenopausal females. Baseline serum ferritin concentrations were strongly associated with hypertriglyceridemia component of metabolic syndrome at the end of the follow-up period (431)

Park et al. conducted a prospective cohort study in 2012, following 18.022 Korean men participating in a medical health check-up program who were free from metabolic syndrome at baseline for a period of 5 years. During 45,919.3 person-years of follow-up, the authors observed an increased risk of developing metabolic syndrome across serum ferritin quintiles: HR_{Q2} = 1.19 (95%CI: 0.98-1.45), HR_{Q3} = 1.17 (95%CI: 0.96-1.43), HR_{Q4} = 1.36 (95%CI: 1.12-1.65), and HR_{Q5} = 1.66 (95%CI: 1.38-2.01, $P_{trend} < 0.001$) (432).

Xiao et al. were among the first groups to publish a case-control study examining the association between iron and metabolic syndrome. The group age matched 87 individuals with metabolic syndrome to 102 healthy adults were recruited from the same

region in China. The authors grouped participants into dichotomous categories of dietary iron intake (i.e., < 15 mg/d vs. ≥ 15 mg/d) and reported an increased risk of metabolic syndrome in cases whose intake exceed 15 mg/d (OR = 7.12, no 95%CI provided). When stratified by type of iron intake (i.e., heme iron intake vs nonheme iron intake) the authors reported an increased risk of metabolic syndrome among those consuming > 7.5 mg of heme iron per day (OR = 7.73, no 95%CI provided) (433)

A year later, Hamalainen et al. conducted a population based cross-sectional study using data obtained from 766 Caucasian, middle-aged subjects (341 males and 425 females) from Pieksämäki, Finland who were recruited for a free health check-up in 2004. The authors reported that both hemoglobin and serum ferritin concentrations were higher in those with metabolic syndrome ($P < 0.001$ (hemoglobin), $P = 0.018$ (ferritin)), noting that hemoglobin concentration was higher in those with any metabolic syndrome component ($P < 0.001$) and that serum ferritin was higher in those with abdominal obesity ($P < 0.001$), high triglycerides ($P = 0.002$) or elevated glucose concentrations ($P = 0.02$) (434)

Finally, several cross-sectional studies have highlighted an association between iron stores and metabolic syndrome. For example, in a population-based cross-sectional study using subjects participating in NHANES III between 1988 and 1994, Jehn et al. found that metabolic syndrome was more common in those in the highest quartile of serum ferritin concentration compared to those in the lowest quartile of serum ferritin concentration in males (27.3% vs. 13.8%; $P < 0.001$), pre-menopausal females (14.9% vs. 6.4%; $P = 0.002$), and post-menopausal females (47.5% vs. 28.2%; $P < 0.001$) (12).

In 2005, Bozzini et al. reported increased odds of developing metabolic syndrome among cases (n = 269) compared to age-matched counterparts who did not have metabolic syndrome (n = 210): OR = 1.53 (95%CI: 1.27-1.84) (15). During that same year Choi (2005) examined the association between serum ferritin, alanine aminotransferase (ALT) levels and cardiovascular risk factors of metabolic syndrome among 959 postmenopausal Korean females. The authors reported that cases demonstrated higher serum ferritin (74.7 ± 2.0 ng/mL vs 59.6 ± 2.0 ng/mL, $P < 0.001$) and ALT levels (21.3 ± 1.6 IU/L vs 18.7 ± 1.5 IU/L, $P < 0.001$). Moreover, increasing concentrations of serum ferritin corresponded to the presence of a greater the number of metabolic syndrome components ($P < 0.001$) (435).

Similar to the results reported by Choi et al., in 2008 Shi et al. observed an increasing number of metabolic syndrome components with increasing serum ferritin concentrations in their study sample. Moreover, the authors reported that individuals with insulin resistance had increased serum ferritin concentrations compared to individuals without insulin resistance (124.5 ng/mL vs 80.1 ng/mL, $P < 0.001$). In addition, the authors noted that serum ferritin concentrations were higher among individuals with triglyceride concentrations ≥ 150 mg/dL compared to those whose triglyceride concentrations were < 150 mg/dL (76.8 ng/mL vs 40.1 ng/mL, $P < 0.001$) and higher among those with fasting plasma glucose concentrations ≥ 110 mg/dL compared to those whose fasting plasma glucose concentrations were < 110 mg/dL (75.7 ng/mL vs 41.7 ng/mL, $P = 0.005$) (436).

Later that year, Sun et al. conducted a cross-sectional survey using data obtained from 3,289 participants aged 50 to 70 years residing in Beijing and Shanghai China,

reporting an increased risk of metabolic syndrome for those in the highest quartile of serum ferritin concentrations compares to those in the lowest quartile of serum ferritin concentrations (OR 2.80 (95%CI: 2.24-3.49)) after adjustment for other dietary exposures, BMI, inflammatory markers, and adipokines (392).

Ryu (2008) conducted a cross-sectional study of 1,444 adults between the ages of 40 and 70 participating in a survey conducted as part of the Korean Rural Genomic Cohort Study (KRGCS), reporting that the prevalence of metabolic syndrome was higher among those in the highest quartile of serum ferritin concentration compared to those in the lowest quartile of serum ferritin concentration (37.1% vs. 22.4%, $P = 0.006$) in males and (58.8% vs. 34.8, $P < 0.001$) in females. Moreover, the authors observed an increased risk of metabolic syndrome among those in the highest quartile of serum ferritin concentration compared to those in the lowest quartile of serum ferritin concentration (OR = 2.21 (95%CI: 1.26-3.87, $P = 0.024$) in males and OR = 2.10 (95% CI: 1.40-3.17, $P = 0.001$) in females. In line with the observations of Choi et al. and Shi et al., the authors noted that increasing serum ferritin concentrations corresponded to a greater number of metabolic syndrome components (437).

In a smaller cross-sectional study of 482 Tehrani females, subjects in the highest quintile of red meat intake (i.e., ≥ 63.7 g/d) had greater odds of metabolic syndrome (OR = 2.33; 95%CI: 1.24-4.38; $P_{trend} < 0.01$) (14). The aforementioned group found a similar association using the same subjects classified according to dietary patterns, where females in the highest quintile of Western diets (i.e., characterized by high red-meat and refined grain intakes) had increased odds of metabolic syndrome (OR = 1.68; 95%CI: 1.10-1.95; $P_{trend} < 0.01$) (14). As previously mentioned, a limitation of a cross-sectional

study design is that one cannot decipher whether increases in body iron occurred before, during or after the development of metabolic syndrome.

Kim (2011) investigated the association of serum ferritin concentrations with insulin resistance and impaired glucose metabolism among 12,090 subjects (6378 men and 5712 females between the ages of 20-89 years) undergoing general medical checkups at the Health Promotion Center at Asan Medical Center in Seoul, Korea. The study sample was comprised of 7,253 participants with NGT, 3,783 participants with IGT and 1,054 participants with T2DM. The authors reported an increased risk of metabolic syndrome among those with NGT when in the highest quartile of serum ferritin concentrations compared to those in the lowest quartile of serum ferritin concentrations (OR = 2.85 (95%CI: 1.99-4.07)) in males and OR = 1.21 (95%CI: 0.82-1.79)) in females (393).

Kang (2012) examined data obtained from 7,346 individuals (i.e., 3229 males, 4,117 females) participating in the 2007-2008 Korean National Health and Nutrition Examination Survey (KNHANES). The authors reported an increasing risk of metabolic syndrome among those in the highest quartile of serum ferritin concentrations compared to those in the lowest quartile of serum ferritin concentrations (OR = 1.67, 95%CI: 1.24-2.23 in males and OR = 1.41, 95%CI: 1.06-1.88) in females after adjusting for menopausal status. Moreover, the authors also observed that increasing serum ferritin concentrations were associated with components of metabolic syndrome, such as increased triglyceride concentrations in males and glucose intolerance in females (7).

During the same year, Gabrielson et al. followed a group of 110 individuals to investigate the effect of iron intake on adiponectin, which is an insulin-sensitizing

adipokine that is commonly decreased among those with metabolic syndrome. The authors reported that serum ferritin concentrations were increased and adiponectin concentrations were decreased among those with metabolic syndrome compared to those without metabolic syndrome (260 ± 23 ng/mL vs 185 ± 21 ng/mL) (438).

In another population-based cross-sectional study conducted in Taiwan, Chang et al. found that individuals in the highest tertile of serum ferritin concentrations had a higher risk of metabolic syndrome compared to those in the lowest tertile of serum ferritin concentrations (OR = 1.72, 95%CI: 1.21-2.45) (16). The authors further reported that serum ferritin concentrations demonstrated a linear relationship with individual components of metabolic syndrome. For instance, individuals in the highest tertile of serum ferritin had higher fasting serum glucose concentrations (OR = 2.16, 95%CI: 1.75-2.65) and higher serum triglycerides (OR = 2.58, 95%CI: 1.07-3.22) (13).

Li et al. (2013) sought to explore the relationship between serum ferritin and metabolic syndrome among 8,441 adults aged 18 and older participating in the China Health and Nutrition Survey. The authors observed that serum ferritin concentrations were significantly higher among those with metabolic syndrome (106.0 ng/mL vs 73.2 ng/ml, $P < 0.001$) after adjustment for age, nationality, BMI, smoking status and alcohol consumption. Finally, the authors noted that the risk for metabolic syndrome increased across increasing quartiles of serum ferritin concentrations for both males and females ($P_{trend} < 0.001$) (9).

Tang et al examined data obtained from 3,274 males participating in the 2009–2013 Fangchenggang Area Males Health and Examination Survey (FAMHES). The authors reported that those with metabolic syndrome had higher serum ferritin

concentrations compared to those without metabolic syndrome (median: 447.4 ng/mL (range: 294.1-612.4) vs. 302.4 ng/mL (range: 215.0-435.8), $P < 0.01$). Moreover, the authors observed positive correlations between serum ferritin concentrations and components of metabolic syndrome: blood pressure (BP) (Systolic BP: r = 0.11, Diastolic BP: r = 0.16), waist circumference (r = 0.33), fasting glucose concentration (r = 0.09), triglyceride concentration (r = 0.32) and low HDL cholesterol (r = 0.13) ($P < 0.001$ for all comparisons). Finally, the authors observed an increased risk of metabolic syndrome among those in the highest quartile of serum ferritin concentrations compared to those in the lowest quartile of serum ferritin concentrations (OR = 2.29, 95%CI: 1.47-3.54) (439).

More recently, two groups have each conducted separate meta-analyses examining the association between iron parameters (i.e., serum ferritin) and metabolic syndrome with many of the above-mentioned studies. In 2014, Abril-Ulloa et al. included a total of fifteen studies in the calculation of pooled effect sizes (i.e., two prospective, one case-control and twelve cross-sectional studies, Table 9) and reported separate pooled effect sizes for both males and females. Overall, the pooled effect size across studies comparing the highest versus the lowest category of serum ferritin among study participants with metabolic syndrome was 1.73 (95%CI: 1.54-1.95, I-squared = 75.4%). For males, the pooled effect size for the association between the highest reported category of ferritin across studies to that of the lowest category of ferritin was 1.69 (95%CI: 1.29-2.21). Among females, the pooled effect size was 1.65 (95%CI: 1.41-1.94). A strength of the meta-analysis was that thirteen of the fifteen studies included used the NCEP-ATP III definition to determine the presence of metabolic syndrome. Moreover, the authors were able to conduct subgroup analyses among studies adjusting for C-

reactive protein (CRP) and reported a pooled effect size among studies controlling for CRP of 1.92 (95%CI: 1.61-2.30) compared to 1.52 (95%CI: 1.36-1.69) for studies not controlling for CRP. This finding is interesting, as one would expect the effect size to be attenuated by controlling for factors related to inflammation (11).

Jin et al. published another meta-analysis reporting upon the association between serum ferritin and metabolic syndrome in 2015. In contrast to the former group, Jin and colleagues reported pooled effect sizes among studies examining the association between mean ferritin concentrations in study participants with metabolic syndrome compared to mean ferritin concentrations among study participants without metabolic syndrome. The authors included eight studies (four prospective, two case-control and two cross-sectional studies, Table 9), none of which overlapped with those analyzed by Abril-Uloa et al. in their meta-analysis, and reported a pooled effect size of 1.20 (95%CI: 0.69-1.71, I-squared = 96%) (440) comparing the standardized mean difference in serum ferritin concentrations among study participants with metabolic syndrome to those without metabolic syndrome. It is plausible that the reported effect size was not statistically significant due to the high reported heterogeneity across studies, the inconsistent application of criteria to determine the presence of metabolic syndrome and the scaling of the dependent variable (i.e., continuous vs categorical) among the studies included in the meta-analysis. Despite these limitations, the reported effect size is in the same direction and of similar magnitude to those reported by Abril-Uloah.

Interventional Epidemiology Additional support for a positive association between body iron and metabolic syndrome has been obtained from an interventional study published by Houschyar et al. in 2012. The authors randomly allocated 64

individuals with metabolic syndrome to a treatment arm (i.e., iron reduction by phlebotomy, N = 33) or control arm (N = 31). Those in the treatment arm had 300 mL of blood removed at study entry and, depending upon baseline serum ferritin concentration, between 250 mL and 500 mL removed after four weeks. The primary outcomes of the study at the end of six weeks were 1) change in systolic blood pressure (SBP) and 2) change in HOMA-IR. Secondary outcomes measured by the group were 1) change in HbA1C, 2) change in plasma glucose concentration, 3) change in lipid profile and 4) change in heart rate beats per minute (bpm). The authors observed a significant reduction in SBP in the treatment group at the end of the study period (148.5 ± 12.3 mmHg to 130.5 ± 11.8 mmHg, representing a difference of -16.6 mmHg (95%CI: -20.7 to -12.5, $P < 0.001$), which corresponded to a reduction in serum ferritin concentrations in the treatment group from 188.3 ± 212.4 to 104.6 ± 132.5. While those in the control group also experienced a reduction in serum ferritin concentrations from 173.2 ± 132.9 to 149.4 ± 124.9, this was not statistically significant. The average between-group reduction in serum ferritin concentration was 74.2 ng/mL ($P < 0.001$), favoring the treatment group. The change in SBP among those in the control arm was not significant (144.7 ± 14.4 mmHg to 143.8 ± 11.9 mmHg). While HOMA-IR decreased in both arms (treatment HOMA-IR: 4.8 ± 7.2 to 3.6 ± 2.7; control HOMA-IR: 4.5 ± 3.8 to 4.1 ± 3.6), neither reduction was statistically significant. The greater reduction in HOMA-IR in the treatment arm, however, correlated with serum ferritin reduction ($r = 0.39$, $P = 0.03$). Finally, those in the treatment arm experienced significant reductions in all secondary outcomes (i.e., decreased HbA1C (from 5.56 ± 0.61 to 5.36 ± 0.58, $P < 0.001$)), blood glucose concentrations (from 110.7 ± 29.4 to 98.5 ± 24.0, $P < 0.001$), LDL/HDL ratio

(from 2.48 ± 0.88 to 2.21 ± 0.80, $P < 0.01$) and heart rate (from 72.1 ± 8.2 bpm to 70.0 ± 5.8 bpm, $P < 0.001$) (28).

Conclusion Taken together, it appears as though iron stores are positively associated with the development of metabolic syndrome, including several individual components of metabolic syndrome (e.g., visceral adiposity, dyslipidemia, hypertension, as well as with increasing fasting insulin concentration and blood glucose levels). It should be noted, however, that each of the above studies used serum ferritin and/or transferrin saturation as indices of iron stores. As already mentioned, these parameters demonstrate diurnal variation and/or can be influenced by inflammation. Therefore, predictive, non-invasive, and inexpensive analytes unaltered by inflammation would be valuable for use in monitoring the iron status of those at risk for developing metabolic syndrome. To my knowledge, no study to date has employed the algorithm developed by JD Cook et al. in 2003 (50) as an index for iron repletion to examine the association of the former with metabolic syndrome.

Epidemiology of non-alcoholic fatty liver disease (NAFLD)

History & Definition Non-alcoholic fatty liver disease is a chronic disease that spans a spectrum of hepatic pathology ranging from simple steatosis (i.e., defined as greater than 5% of total liver weight due to an accumulation of triglycerides inside hepatocytes, in the absence of hepatitis B and C virus or excessive ethanol intake) (441, 442) to steatohepatitis, fibrosis and cirrhosis (443, 444). Studies whose aim has been to quantify the prevalence of NAFLD have been hindered by the fact that individuals at any stage of the disease are relatively asymptomatic (444) and techniques/tools (e.g.,

ultrasonography, ALT assay, aspartate aminotransferase (AST) assay) which are commonly used in a clinical setting to diagnose the condition are neither sensitive nor specific (Table 7). Moreover, the gold standard for diagnosing and staging NAFLD is a liver biopsy (444), which is invasive and often cost-prohibitive at both the patient- and population-level.

Descriptive Epidemiology Before investigators in NHANES III (1988-1994) began performing gallbladder ultrasonography on all participants aged 20-74 years of age, there was no representative data in the U.S. available to study the epidemiology of NAFLD (442). These studies, however, revealed that the prevalence of NAFLD in the U.S. increased from 18% from 1988-1991 to 29% in 1999 (35). However, more recent and representative prevalence estimates of NAFLD using more sophisticated imaging techniques emerged from the Dallas Heart Study (DHS). The DHS was a multi-ethnic population-based cohort which collected data from July 2000 through January 2002 (446). During this time period, 2,287 participants were screened using proton magnetic resonance spectroscopy to measure fat content of the liver. Based upon these data, the prevalence of NAFLD in the U.S. in 2002 was estimated to be 30%. Supporting these imaging data are observational studies which report that the prevalence of NAFLD tends to closely parallel the prevalence of obesity (442). Given that ~1/3 of the U.S. population is considered obese, the prevalence of NAFLD in the U.S. population is thus likely to be approximately 30% (446).

Due to the asymptomatic presentation of NAFLD, most cases are diagnosed in the 5th and 6th decades of life (443). NAFLD is rarely observed in individuals younger than

20 years (~1%), but increases to 18% in those aged 20-40 years and to 39% among those aged 60 and older (442).

The prevalence of NAFLD varies across genders and ethnicities. Prior to the aforementioned population-based studies, NAFLD was traditionally thought to affect more females than males (442). However, authors have since demonstrated that males are twice as likely to develop NAFLD than females (441). For example, NHANES data indicate that the prevalence of NAFLD in U.S. males is estimated to be 53.1% versus 29.1% in females (442). In addition, data from the Modena and Bologna/Turin study indicates that NAFLD exhibits a gender dimorphic pattern in which males tend to develop the disease approximately 10 years before their female counterparts (447). Interestingly, postmenopausal females are twice as likely as premenopausal females to develop NAFLD (444, 448). Moreover, the prevalence of NAFLD in females exceeds that in males following menopause (447).

Data from the DHS indicates that the prevalence of NAFLD in Hispanic Americans is 45%, 33% in non-Hispanic whites and 24% in African Americans (443). Moreover, data from several other population-based studies are in alignment with the DHS, showing that Hispanic Americans demonstrate a higher prevalence of NAFLD compared to non-Hispanic whites and African Americans across all age categories and genders (442, 446). Explanations for the observed differences in the prevalence of NAFLD are hypothetical and relatively vague. Moreover, some authors posit that the observed differences may be related to the ethnic differences in body fat distribution and/or fat metabolism, with African Americans having more subcutaneous fat but less visceral fat. It has also recently been suggested that African Americans have different

lipoprotein metabolism (i.e., lower serum concentrations of triglycerides than age and gender-matched counterparts) compared to other ethnicities (449). Lastly, the higher observed prevalence of NAFLD in Hispanic Americans has also been attributed to intrinsic factors such as a genetic predisposition for greater adiposity and insulin resistance compared to other ethnicities (442).

Pathophysiology Several studies have reported that NAFLD is highly correlated with obesity, T2DM (442, 444), biomarkers of dyslipidemia (e.g., high triglyceride concentrations and low HDL concentrations) and insulin resistance. However, insulin resistance appears to be considered the pathophysiological hallmark of NAFLD (442), as mild insulin resistance/hyperinsulinemia is commonly observed at the earliest stages of NAFLD in the absence of obesity (450) and more severe insulin resistance (e.g., such as that observed in metabolic syndrome and T2DM) correlates with more advanced stages of NAFLD (442).

To my knowledge, a single hypothesis has been put forward to explain the etiology of NAFLD, which highlights the role of increased concentrations of FFAs leading to insulin resistance and subsequent hepatic steatosis. In the normal hepatocyte, energy received from circulating glucose, fructose and lipids is stored as glycogen and triglycerides, respectively. Under normal physiologic conditions, any increase in lipid concentration within the hepatocyte results in 1) the redistribution of the lipids to peripheral storage within adipose tissue or 2) the lipids are directly converted to metabolic fuel through oxidation. In other words, it appears that hepatocytes have very little capacity for the storage of triglycerides (i.e., the storage form of FFAs) and do so only when all other mechanisms for lipid utilization, trafficking or storage have been

exhausted. In NAFLD, lipid trafficking appears to be dysregulated, which culminates in increased intrahepatocellular lipids, particularly as saturated FFAs (e.g., palmitic acid, stearic acid) (451).

Within the hepatocyte, these excess FFAs appear to directly contribute to organelle dysfunction and failure. As mentioned previously, FFAs contribute to mitochondrial dysfunction by skewing the NAD/NADH ratio which leads to increased concentrations of acetyl-CoA and hepatic glucose production. Over time, this culminates in elevated triglyceride storage within the hepatocyte, organelle failure and apoptosis. Moreover, FFAs can induce endoplasmic reticulum stress by increasing ceramide concentration (452) as well as by other poorly understood mechanisms (452).

There is also substantial evidence which demonstrates that FFAs can directly induce cellular toxicity by increasing oxidative stress and activation of pro-inflammatory pathways (457). For example, FFA accumulation within the hepatocyte is associated with Kupffer cell activation (452). Kupffer cells are resident macrophages within the liver parenchyma in close physical proximity to hepatocytes, endothelial cells, sinusoids and stellate cells (452). It is worth noting that stellate cells reside between hepatocytes and the small blood vessels of the liver and are normally quiescent. It appears that their main function is to store vitamin A and maintain the basement membrane matrix. Activation of stellate cells leads to collagen secretion and formation of scar tissue and appears to be a central step in the initiation of hepatic fibrosis. Interestingly, not only is the activation of Kupffer cells thought to play a central role in the pathogenesis and progression of liver disease by contributing to parenchymal inflammation, hepatocyte ballooning and injury

and initiation of fibrosis via TNF-alpha secretion (452), Kupffer cells have also been shown to modulate stellate cell activation (454)

In terms of linking FFAs to inflammation and NAFLD, TNF-alpha secretion by macrophages promotes lipolysis and increases FFA concentration (455) and has been observed to increase in a stepwise-fashion from obesity to simple steatosis (452). Interestingly, TNF-alpha both promotes and is activated by insulin resistance via activation of IKK-B (inhibitor of nuclear factor kappa B kinase subunit beta (NF-kB)) (456), which mediates the activity of NF-kB (i.e., a proinflammatory protein complex). This complex regulates inflammatory mediators including C-reactive protein (CRP), plasminogen activator inhibitor (PAI-1), TNF-alpha and IL-1B (456).

As mentioned previously, TNF-alpha has been shown to antagonize adiponectin (452) and vice versa (457). Thus, it has been postulated that adiponectin may protect the liver from inflammation via this antagonistic effect on TNF-alpha. Lending support to this line of reasoning, recombinant adiponectin has been shown to inhibit TNF-alpha concentration, improve insulin sensitivity and reduce steatohepatitis in two mouse models of hepatic steatosis (452). Taken together, these results support the hypothesis of FFAs leading to insulin resistance and subsequent NAFLD, where FFA accumulation leads to insulin resistance and subsequent hepatic steatosis, which is amplified by increasing insulin resistance.

Analytic Epidemiology The risk for developing NAFLD correlates positively with BMI, as prevalence of the disease ranges from 30% in normal weight individual of the general population to 57% among overweight individuals (446) to 80%-90% among

obese individuals (449). Given that 66% of U.S. population is estimated to be overweight (i.e., as indexed by BMI), this is obviously a growing public health concern.

Suzuki et al. have speculated that FFA oxidation in the liver may decrease following menopause while lipogenesis may be enhanced (447). Thus, it appears that menopausal status (i.e., pre- vs. post-menopause) be considered a risk factor. In line with this observation, other authors report that postmenopausal females receiving hormone replacement therapy are significantly less likely to develop NAFLD compared to females who do not (442, 447) and Carulli et al. have postulated that circulating levels of estrogens within the physiological range might be responsible for a protective effect on the development of hepatic steatosis (458). In support of the latter hypothesis, NAFLD in animal models has been reversed by the administration of estradiol (459). Interestingly, it appears that no connection has been made between the development of NAFLD among pre- and post-menopausal females and the cessation of menstruation among postmenopausal females.

Given that much of the research community concurs that NAFLD is the 'hepatic manifestation of metabolic syndrome,' additional risk factors independent from those already associated with the development of metabolic syndrome are not commonly reported in the NAFLD literature. Similar to T2DM and Metabolic syndrome, however, several small case studies have indicated that parameters of iron status may be involved in the initiation, promotion and progression of NAFLD.

Iron and non-alcoholic fatty liver disease Given that parameters of iron status and their association with NAFLD have received little attention in the epidemiological literature, it follows that presenting the results of investigations relating the risk of

developing NAFLD to iron stores in a systematic manner is a challenge. Among the studies examining the relationship between iron and NAFLD, dysmetabolic iron overload syndrome (DIOS) appears to be a common feature of NAFLD, with a prevalence of ~33% (460) among those diagnosed with the condition. In subjects with DIOS, serum ferritin is elevated, while transferrin saturation is within the normal reference range (461).

As mentioned, associations between iron stores and NAFLD have been most often reported in case-studies. For example, in 2004, Younossi et al. obtained biopsies from 65 patients with NASH, noting that 31% of patients were either heterozygous or homozygous for the C282Y polymorphism at the HFE locus rs1800562, and the presence of this mutation resulted in a higher histological grade of iron, increased hepatic iron content and an increase in histological fibrosis (462). Gene expression data published by Mitsuyoshi et al. in 2009 support the results of Younossi et al. The former group obtained biopsy specimens from 74 patients with NAFLD and observed an increase in hepatic iron score (HIS) as the stage of fibrosis progressed. Moreover, sTfR1 expression significantly increased with progressing stage of fibrosis (463). In addition, Nelson et al. published a study in 2011 with data obtained from 849 patients enrolled in the Nonalcoholic Steatohepatitis Clinical Research Network demonstrating that participants whose reticuloendothelial system (RES) cells (i.e., monocytes and macrophages) contained stainable iron were also more likely to have advanced pathohistological features such as fibrosis, portal inflammation, hepatocellular ballooning and biopsy-confirmed NASH compared to participants without stainable hepatocellular iron. In this study, the presence of stainable RES iron was independently associated with advanced hepatic fibrosis following multiple regression analysis controlling for age, gender, diabetes status, and

BMI (OR = 1.60, 95%CI: 1.10 – 2.33, *P* = 0.015) (464). In 2012, Shim published a study showing that elevated serum ferritin was an independent predictor of histologic severity and advanced fibrosis in subjects with NAFLD (465). Later that year another group (466) conducted a genetic association study examining the effects of the C282Y (rs1800562) and H63D (rs1799945) polymorphisms on serum hepcidin, hepatic iron deposition, and histology of NAFLD. The authors observed that subjects with NAFLD and C282Y (but not H63D) mutations had lower median serum hepcidin levels and higher mean hepatocellular iron grades compared to wild-type subjects. Nelson et al. also observed an inverse relationship between hepatic iron stores and serum hepcidin concentration across all *HFE* genotypes, indicating that elevated iron stores are associated with NAFLD independent of *HFE* genotype (466). The latter observation is supported by a study conducted in 1999 by Mendler et al. who reported that patients with no *HFE* mutations had similar degrees of iron overload as those with mutations in the *HFE* gene, apart from compound heterozygotes (i.e., individuals heterozygous for both *HFE* mutations), who presented with significantly more iron burden (467). A meta-analysis published in 2012, which includes the study conducted by Mendler et al., lends support to these individual investigations, as the authors concluded that elevated iron in NAFLD is independent of *HFE* genotype (468). It thus appears that insulin resistance in NAFLD is associated with more subtle degrees of iron overload than is observed in iron-loading disorders, such as HH and beta-thalassemia (469).

Mechanistically, iron appears to have a direct role in the activation of hepatic Kupffer cells and hepatic stellate cells. For example, iron has been shown to activate inflammatory signaling via hepatic macrophages *in vitro* and ferritin treatment of isolated

rat hepatic stellate cells has been shown to increase NF-kB signaling. As mentioned, the activation of stellate cells appears to be a central step in the initiation of hepatic fibrosis. Animal studies have also shown that hepatic sTfR-1 is upregulated due to a high-fat diet, which may lead to increased hepatocellular uptake of dietary iron despite an already increased hepatocellular iron load. In alignment with these studies, Otogawa et al have observed an increase in red cell fragility in response to a high-fat diet in rabbits and this fragility leads to increased erythrophagocytosis by Kupffer cells (470). From a pathophysiological perspective, Kupffer cells can also accumulate iron via phagocytosis of necrotic hepatocytes (471). These latter findings may, in part, explain the observed increases in hepatic reticuloendothelial iron that has been observed in some NASH cohorts (154).

Serum hepcidin levels also appear to increase as individuals transition from NAFLD to NASH, and DMT-1 is upregulated in patients with NASH, despite the observed increased serum hepcidin concentrations (472), indicating a perturbation in iron metabolism where the requirement for iron is increased along with iron storage.

Finally, dietary iron loading in leptin-receptor deficient mice has been recently reported to lead to inflammasome and immune cell activation, which directly corresponds to hepatocellular ballooning. In other words, mice who lack satiety signals and have high intakes of dietary iron appear to have Kupffer cell activation and increased oxidative stress, which leads to liver dysfunction.

Interventional Epidemiology Valenti et al. demonstrated in a case-control study that iron depletion by phlebotomy improves insulin resistance in patients diagnosed with NAFLD (473). More recently, a meta-analysis of studies examining the effect of

phlebotomy on insulin resistance lends further support to the initial results of Valenti et al. The primary outcome analyzed across all studies in the meta-analysis conducted by Jaruvongvanich et al. (Table 10) was the pooled mean difference (MD) of the homeostasis model assessment of insulin resistance (HOMA-IR) (468). The authors found that study subjects with NAFLD who underwent phlebotomy had lower HOMA-IR indices with an MD of 0.84 (95%CI: 0.01 to 1.67, I-squared = 34%), lower triglyceride levels (MD = 9.89, 95%CI: 4.96-14.83, I-squared = 22%) levels and increased high density cholesterol level (MD = 3.48, 95%CI: 2.03-4.92, I-squared = 18%) compared to controls (i.e., subjects with NAFLD who did not receive phlebotomy) and concluded that phlebotomy decreased insulin resistance and liver transaminase levels in patients with NAFLD.

Conclusion Taken together, it appears as though iron stores are positively associated with NAFLD. Compared to T2DM and metabolic syndrome and a lack of several population-based studies, however, the strength of the associations between iron stores and NAFLD are unclear. Moreover, each of the above studies employed invasive and costly methods to assay iron status. Predictive, non-invasive, and inexpensive analytes unaltered by inflammation would be valuable for use in monitoring the iron status of those at risk for developing NAFLD. To my knowledge, no study to date has employed the algorithm developed by JD Cook et al. in 2003 (50) as an index for iron repletion to examine the association of the former with NAFLD.

Given that the prevalence of NAFLD is increasing and that quantification of the presence of the condition often involves cost-prohibitive and/or invasive methods, an index with high sensitivity and specificity which utilizes biochemical parameters would

be of significant value. To this end, several indices and algorithms have been developed. However, the sensitivity and specificity of each index varies (Table 7) in terms of accurate classification across populations. Thus, an index referred to as the U.S.-FLI has was recently developed to determine the presence of NAFLD among multi-ethnic cohorts. As mentioned, the index accounts for age and ethnicity and has been shown to outperform its predecessor, the FLI (35).

The U.S.-FLI has not been employed thus far to estimate the prevalence of NAFLD in U.S. females as a subgroup. Moreover, no investigations to date have examined the association between body iron as indexed by Cook's equation and NAFLD as indexed by the U.S.-FLI.

Epidemiology of insulin resistance

History & Definition It is well-established in the literature that insulin resistance is involved in the development of T2DM (366-398), metabolic syndrome (7,9,12-15, 392, 393, 431-440) and NAFLD (460-473), often decades before glucose intolerance occurs or any of the above conditions are diagnosed. Insulin resistance has traditionally been framed in terms of the effects of insulin on glucose metabolism, otherwise known as the "glucocentric" view of insulin resistance. The traditional "glucocentric" view of insulin resistance has shifted over the past decade, however, to a "lipocentric" view of insulin resistance due to advances in our understanding of the etiology of insulin resistance. The "lipocentric" view of insulin resistance will be discussed subsequently.

Descriptive Epidemiology In the U.S. population, the prevalence of insulin resistance has been estimated at 45% (9.6% in normal weight adults aged 40-79 years,

and ~ 50% in overweight adults aged 40-79 years) (474). Prevalence estimates for insulin resistance may be underreported, however, as the gold-standard for diagnosis of insulin resistance is the hyperinsulinemia-euglycemic clamp, which is both invasive as well as cost-prohibitive at the patient-level as well as in population-based studies. In addition, the prevalence of insulin resistance alone has rarely been quantified in the absence of T2DM, metabolic syndrome or NAFLD.

Pathophysiology As previously mentioned, however, the traditional "glucocentric" view of insulin resistance has shifted over the past decade to a "lipocentric" view of insulin resistance due to results emerging from recent investigations. For example, *in vivo* research conducted using 13C-MRS (13C-Magnetic Resonance Spectroscopy) to examine the cellular mechanisms of insulin resistance in humans is a technique which allows for the real-time, non-invasive measurement of the concentration of intracellular metabolites containing naturally occurring isotopes of 1H, 13C, and 31P (475, 476). Using this approach with sets of age- and weight-matched subjects with and without T2DM, Peterson and colleagues have demonstrated that the rate of insulin-stimulated muscle glycogen synthesis (i.e., assessed via the incorporation of 13C-glucose into gastrocnemius/soleus muscle glycogen) is decreased by ~50% in T2DM subjects (475, 476). Extrapolating these data to the whole-body, the group concluded that almost all the insulin resistance observed in these subjects could be attributed to defects in insulin-stimulated muscle glycogen synthesis. Further analysis of the glycogen synthesis pathway revealed that the rate-limiting step leading to insulin resistance was reduced insulin-stimulated glucose transport activity. Finally, through additional 13C and 31P MRS studies, the group determined that the accumulation of

FFAs within the myocyte induces insulin resistance (i.e., via reducing insulin action) by directly inhibiting activation of glucose transport activity by reducing transcription of GLUT4 (476), the insulin-regulated glucose transporter primarily involved in regulating whole-body glucose homeostasis.

To appreciate the impact of FFAs on GLUT4 transcription, we must examine the tissue distribution and mechanism of action of GLUT4. The tissues which remove glucose from circulation for utilization via GLUT4 are skeletal muscle, liver and adipose tissue. Under normal physiologic conditions, insulin secretion 1) stimulates glucose uptake in skeletal muscle and adipocytes by translocation of GLUT4 to the cell surface, 2) stimulates the synthesis of glycogen from glucose and inhibits glycogenolysis in skeletal muscle and hepatocytes and 3) decreases hepatic gluconeogenesis (i.e., the generation of glucose from non-carbohydrate substrates such as amino acids, triglycerides, pyruvate and lactate) (477), in order to prevent an influx of additional glucose into the bloodstream. Moreover, insulin secretion inhibits lipolysis (i.e., fat breakdown) in adipocytes and stimulates glucose uptake in adipose tissue (478). Thus, the net effect of insulin secretion by beta cells is to increase glucose uptake via GLUT4, reduce circulating glucose levels by suppressing hepatic glucose production and to increase the conversion of glucose to glycogen or fat (477).

Experimental models have demonstrated that altering GLUT4 activity directly impacts insulin action. For example, mice with targeted downregulation of GLUT4 in skeletal muscle and adipose tissue have been shown to develop insulin resistance in each tissue (478). Moreover, it has also been shown in mouse models that overexpression of GLUT4 in adipose tissues is associated with improved insulin sensitivity (480). Taken

together, it appears that the "lipocentric" view of insulin resistance usurps the previous "glucocentric" view of insulin resistance, as the former view places FFAs at the beginning of the pathway which leads to beta cell dysfunction by decreasing GLUT4 expression and this is thought to result in hyperglycemia with subsequent hyperinsulinemia as a compensatory response.

It is worth noting that with increasing FFAs, it has been postulated that glucose oxidation (as well as glycolysis) is reduced in favor of FFA oxidation. As a consequence, the metabolic by-products of excess FFA oxidation (i.e., ceramides) (452) contribute to oxidative stress, which is thought to lead to further increases beta cell dysfunction and apoptosis (333). For instance, studies have shown that ceramide directly impairs insulin-stimulated glucose uptake and GLUT4 translocation when added to cultured adipocytes and myocytes (481). These effects appear to be mediated by the effects of ceramide to inhibit tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1, which transmits signals from insulin to downstream intracellular pathways such as PI3K/Akt and MAP kinase) (478). Moreover, FFAs are endocytosed and metabolized directly by beta cells (477), which has been hypothesized to lead directly to beta cell dysfunction (477). Moreover, FFAs can be re-esterified to triglycerides by several tissues including muscle, fat and liver (341), which leads to the increased storage of these lipids.

As mentioned in the section covering T2DM, studies have demonstrated that FFAs contribute to mitochondrial dysfunction by skewing the NAD/NADH ratio and this is thought to lead to inhibition of FFA beta oxidation, increases in FFA storage as triglycerides, mitochondrial dysfunction and eventual mitochondrial apoptosis. Thus, the manifestation of diminished GLUT4 activity via increased FFA concentrations, coupled

with increasing hyperinsulinemia and hyperglycemia, results in a variety of metabolic abnormalities including several perturbations downstream of insulin resistance. These perturbations manifest in hepatic steatosis (previously discussed in the NAFLD section), elevated triglycerides, low levels of high-density lipoproteins (HDL) and increased secretion of very low-density lipoproteins (VLDL) (previously discussed in the metabolic syndrome section).

Iron & insulin resistance In a three-year prospective study among 1,277 French adults, Fumeron et al. reported that baseline serum ferritin level was an independent predictor of increases in serum insulin concentrations at the end of the follow-up period. The authors reported that each 1 ng/mL increase in ferritin was associated with an 0.05 ± 0.02 µU/mL increase in serum insulin concentration ($P =$ 0.002) (373). Two other cross-sectional studies have indicated that increased body iron is associated with insulin resistance.

The first cross-sectional study examined the effect of dietary patterns (i.e., healthy, Western, traditional) on iron status and insulin resistance in 486 Tehrani females. In these subjects, a Western dietary pattern classification scheme was developed using factor analysis, where red meats and refined grains had the highest factor loadings of 0.56 and 0.66, respectively (14). The authors observed that females who were in the highest quintile of Western dietary pattern scores (i.e., the highest red meat and refined grain intake) had greater odds of insulin resistance, as measured by the homeostasis model assessment-insulin resistance (HOMA-IR) (OR = 1.26; 95%CI: 1.00-1.78; $P_{trend} < 0.01$). Given the cross-sectional study design, however, it is not possible to conclude whether

increased body iron preceded increases in HOMA-IR, accompanied increases in HOMA-IR or followed increases in HOMA-IR.

In another population-based cross-sectional study involving 1,013 Finnish men, subjects in the highest quintile of serum ferritin concentrations had 21.6% higher fasting serum insulin concentrations (95%CI: 7.3%-37.9%; $P < 0.001$) (24). In these subjects, fasting blood glucose was also higher in those in the highest quintile of serum ferritin concentrations (6.1%; 95%CI: 2.3%-9.9%; $P < 0.001$).

Conclusion Taken together, these three investigations suggest that iron is associated with insulin resistance and may be involved in the etiology of insulin resistance. Additional prospective studies are needed, however, to confirm this hypothesis.

In terms of linking iron to the etiology of insulin resistance by way of a testable hypothesis, we must first examine several lines of evidence demonstrating the effects of insulin administration on iron parameters, as well as the effects of iron on factors reported in the literature to be associated with insulin resistance, such as chemokines, cytokines, adipokines and insulin-sensitizing agents. Most importantly, an association between iron and the purported driver of insulin resistance, FFAs, must be demonstrated.

Effects of insulin administration on iron parameters Two groups thus far have shown that administration of insulin to cultured adipocytes increases iron uptake in these cells by increasing the expression of sTfR1 (482, 483) and decreased insulin-stimulated glucose transport (484). Another group have replicated these findings using the HepG2 hepatic cell line and the HL-7702 hepatic cell line (485). Moreover, these results garner

additional support from a rodent model demonstrating that insulin injections lead to subsequent increases in sTfR1 *in vivo* (486).

Related to these observations, studies have also shown that serum transferrin receptor 2 (sTfR2) can be regulated by insulin-like growth factor 1 (IGF1, a hormone produced in the liver that shares structural similarity to insulin), where IGF1 expression increased sTfR2 cycling (167). Finally, several authors have reported that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can also act as a transferrin receptor and is a preferred receptor for transferrin in some cell lines (CHO-TRVb, J774 cell lines) (77, 78, 487, 488). This is an interesting finding, as GAPDH is the same enzyme involved in the breakdown of glucose to pyruvate during glycolysis (487).

It is also worth mentioning here that the finding from Davis et al. and Tanner et al. is notable, especially when considering other studies which have suggested that iron within the adipocyte might interfere with cellular insulin sensitivity and contribute to increased lipolysis (16). This leads to one to hypothesize that increased lipolysis by iron within the adipocyte could increase the pool of FFAs. Moreover, one can hypothesize that insulin resistance might amplify these effects, as insulin appears to enhance iron uptake by increasing serum transferrin receptor expression with increasing concentrations of insulin.

Iron and chemokines Zager et al. have shown that both iron sucrose as well as iron gluconate increase plasma and renal MCP-1 concentration (489). Given that iron is a pro-oxidant it is feasible that iron could also contribute to increased MCP-1 expression.

Iron and cytokines The effects of iron on the immune system was reviewed previously in chapter 3. However, no special attention was given to the cytokines

associated with the development of insulin resistance nor was any attention given to how iron is associated with insulin resistance or conditions associated with insulin resistance. Several studies have been published which report elevated levels of ferritin and TNF-alpha, IL-6, IL-8 and C-reactive protein (CRP) (380, 382, 490). For instance, in an age- and sex- matched case-control study, Elsammak et al. have reported that both serum ferritin and TNF-alpha increase among individuals with NGT (N = 18), T2DM (N = 22), Hep C infection with T2DM (N = 23) and Hep C infection (TNF-alpha: 0.32 (0-5.8) pg/mL, 0.85 (0-10.5) pg/mL, 19.8 (0.51-139) pg/mL, 25.5 (0.43-124.0) pg/mL, respectively; serum ferritin: 159 ± 76.9 ng/mL, 86.9 ± 41.8 ng/mL, 285.8 ± 124.3 ng/mL, 258.1 ± 116.2 ng/mL, respectively). Moreover, the authors reported that the degree of insulin resistance decreased among those with both HepC infection and T2DM, T2DM, Hep C infection and NGT (HOMA-IR: 6.1 ± 2.36, 4.53 ± 2.84, 3.69 ± 2.2, 1.32 ± 0.49, respectively). In addition, Abou-Shousha et al. have shown that serum ferritin, sTfR and IL-8 are all elevated in states of insulin resistance. Taken together, these results indicate that iron, cytokines and insulin resistance are positively associated and emphasize the importance of excluding and/or controlling for Hep C infection and adjusting for CRP concentrations during the analysis of the effect of iron on any outcome in which insulin resistance plays a role.

Iron and adipokines Leptin and adiponectin are two-well characterized adipokines produced and secreted by adipose. Briefly, leptin was identified in 1994 by Halaas et al. as a 16kDa protein encoded by the *obese* (*ob*) gene. The group discovered the protein to be directly involved in body weight, where its secretion from adipose tissue is activated by the ventromedial hypothalamus of the brain via gastric stretching

following a meal and provides a feedback signal of satiety to the brain so that food intake be halted (491).

Adiponectin was first identified in 1995 by Scherer as 30kDa protein secreted by adipose tissue and is now recognized for its insulin-sensitizing, anti-inflammatory, antiatherogenic and cardioprotective properties (492). Adiponectin secretion is quite distinct from that of leptin. Adiponectin is less dependent on state of satiety compared to leptin. Rather, adiponectin exhibits an ultradian pulsatility (i.e., a pattern characterized by periods of secretion longer than an hour and shorter than a day) and diurnal variation (i.e., adiponectin secretion declines up to 30% during sleep) (493).

Collectively, these adipokines have been shown to decrease triglycerides synthesis, promote FFA degradation (i.e., leptin, adiponectin) (334) and enhance insulin action in both skeletal muscle and liver (i.e., adiponectin) (478). In line with these data, studies which show that leptin concentrations are increased and levels of adiponectin are decreased in obese individuals with insulin resistance as well as in animal models of obesity with insulin resistance (494, 495), suggest that obesity leads to a state of leptin resistance and adiponectin deficiency.

Interestingly, TNF-alpha, whose expression is reported to increase in insulin-resistant conditions, appears to have an antagonistic relationship with adiponectin. Studies have shown that TNF-alpha expression dose-dependently reduces adiponectin expression in adipocytes by suppressing its promoter activity (496). Conversely, adiponectin attenuates the secretion of TNF-alpha from monocytes and macrophages (496). Moreover, increased adiponectin levels appear to reduce TNF-alpha-induced expression of adhesion molecules in vascular smooth muscle cells (VSMCs) (497).

Data published by Green et al. suggests that dietary iron overload induces insulin resistance in mice (484). In their study, the authors concluded that iron accumulation within adipose tissue may result in decreased expression of adiponectin (484). In line with this data, other studies have shown that serum ferritin concentrations are inversely related to adiponectin concentrations (498).

Iron and insulin-sensitizing agents Thiazolidinediones (TZs) are a class of medications also known as the glitazones, which are commonly used to treat T2DM. Administration of TZ has been shown to improve insulin sensitivity (i.e. via lowering circulating glucose and insulin concentrations) by increasing adiponectin gene expression (411) as well as increasing the rate of mitochondrial beta oxidation of FFAs, which decreases FFA concentrations and reduces FFA turnover (411). It has also been suggested that TZs increase mitoNEET expression, a putative direct mitochondrial target for TZs (499). mitoNEET is an iron-sulfur protein residing within the outer mitochondrial membrane (499) and increases in mitoNEET expression have been shown to increase the rate beta oxidation (500), which, as mentioned above, decreases FFA concentrations (411). This leads one to hypothesize that TZs may be involved in balancing the NAD/NADH ratio, as previously discussed.

Iron and FFAs The observation that FFAs are siderophores is based upon a serendipitous discovery in 1991 which began with publications demonstrating that alveolar macrophages in smokers accumulate large amounts of iron (501). Given that macrophages phagocytose senescent erythrocytes, the authors hypothesized that the erythrocytes in smokers were somehow accumulating iron. Although organic extracts of cigarette smoke do not contain significant amounts of iron, previous work by Qian and

Eaton had shown that particulate matter from cigarette smoke contained significant amounts of FFAs. This lead the authors to hypothesize that FFAs bind iron and transfer it to erythrocyte membranes. The authors then used mass spectroscopy to identify the purported iron-binding FFAs in cigarette smoke as strearic and palmitic acid. While Qian and Eaton did not provide data on the definitive structure of the presumed iron:FFA complex, they did hypothesize that the complex forms as a result of the interaction between iron and the carboxylic esters of FFAs (e.g., RCOO:Fe), where the hydrocarbon tails of FFAs are capable of forming a metal chelate, which readily binds iron through an ionic interaction. It therefore became plausible that once erythrocytes had reached the end of their lifespan, they were then phagocytosed by macrophages and this accounted for the observation that alveolar macrophages in smokers had increased amounts of iron.

Nalini and Balasubramanian were the next group to demonstrate that FFAs bind iron, showing that 3 to 4 moles of FFAs are required to bind 1 mole of iron (502). More recently, Boiteau et al. have shown that marine microbes employ the same mechanism to bind iron in conditions of iron scarcity (503). Other authors (504) have gone so far to suggest that iron absorption in the GI tract may also involve FFAs in addition to more widely-known mechanisms of iron absorption, discussed in chapter 2.

Conclusion It appears biologically plausible that iron stores are associated with the initiation, promotion and progression of insulin resistance. Lending support to this hypothesis are studies showing that FFAs are siderophores for iron and that iron activates Kuppfer cells, increases both inflammation and the expression of inflammatory mediators and, as mentioned in Chapter 2, is a pro-oxidant capable of generating ROS and the propagation of lipid peroxidation. Much more population-based research is needed to

tease apart the magnitude of the associations between iron stores and insulin resistance. To this end, predictive, non-invasive, and inexpensive analytes unaltered by inflammation would be valuable for use in monitoring the iron status of those at risk for developing insulin resistance. To my knowledge, no study to date has employed the algorithm developed by JD Cook et al. in 2003 (50) as an index for iron repletion for use in examining the association between the former and insulin resistance.

Summary

The relationships between T2DM, metabolic syndrome, NAFLD and insulin resistance and increased body iron warrants further investigation. Given the iron-replete status of much of the U.S. population, in particular post-menopausal females, it is necessary to increase the sensitivity and specificity of existing estimates of body iron status or to develop novel, non-invasive, and cost-effective procedures to assay individual iron status across the reproductive lifespan.

Predictive biomarkers obtained from serum which correlate with the above mentioned etiological and pathological hallmarks of metabolic dysfunction are of interest in epidemiology and public health due to their value in disease screening, prevention, and treatment. Thus, assays apart from hemoglobin, hematocrit, serum iron, transferrin saturation, total iron binding capacity, or serum ferritin, unaltered by inflammation or liver function would provide increased value in monitoring the iron status of females across the reproductive lifespan.

To my knowledge, the index of iron repletion developed by Cook et al. in 1993 (50), has received no attention in the epidemiological literature with regard to its association with metabolic dysfunction (i.e., Type 2 diabetes mellitus (T2DM) defined by

the World Health Organization (WHO); metabolic syndrome defined by the National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP III); non-alcoholic fatty liver disease (NAFLD) defined by the U.S. Fatty Liver Index (U.S.-FLI); insulin resistance defined by the HOMA-IR) in any population sample.

CHAPTER 5: RESEARCH METHODS

Study population

The study population was comprised of nonpregnant, community-dwelling females aged 12-49 participating in the continuous NHANES 2003-2010 survey with complete data for all parameters of interest. Since 1999, the National Health and Nutrition Examination Survey (NHANES) has collected cross-sectional data every two years on the health and nutritional status of the community-dwelling U.S. population. NHANES data were not obtained using a simple random sample. Rather, the survey obtains a stratified, multistage (i.e., four stages) probability sample designed to represent the U.S. population on the basis of age, sex, and race-ethnicity. The NHANES sampling procedure consisted of four stages. Stage one consisted of primary sampling units (PSUs), which were selected from strata defined by geography and proportions of minority populations. Primary sampling units were mostly single counties or, in a few cases, groups of contiguous counties selected with probability proportional to a measure of size (PPS). Most strata contained two PSUs. Additional stages of sampling were performed to select various types of secondary sampling units (SSUs), namely the segments, households, and individuals that were selected in Stages 2, 3, and 4. In stage two, the PSUs were divided into segments (generally, city blocks or their equivalent). As with each PSU, sample segments were selected with PPS. During stage three, households within each segment were listed, and a sample was randomly drawn. In geographic areas

where the proportion of age, ethnic, or income groups selected for over-sampling is high, the probability of selection for those groups was greater than in other areas. Finally, in stage four, individuals were chosen to participate in NHANES from a list of all persons residing in selected households. Individuals were selected at random within designated age-sex-race/ethnicity screening sub-domains. On average, 1.6 persons were selected per household (505). Because of the differential probabilities of selection, sampling weights were created that reflected the base probabilities of selection, adjustment for non-response, and post-stratification. All subjects have hitherto provided written informed consent and the study was approved by the National Center for Health Statistics Institutional/Ethics Review Board.

Data collection procedures

The current work was a secondary analysis of an ongoing population-based cross-sectional study. Briefly, NHANES data were released for public consumption in 2-year cycles. Just as each year's sample was representative of the resident, community-dwelling U.S. population, the same was true for each 2-year cycle of data. In order to have sufficient sample sizes to obtain stable estimates for population subgroups of interest, however, data cycles spanning the years 2003-2010 were pooled to increase statistical power. Baseline data for continuous NHANES were collected during an in-home interview and a subsequent visit to a mobile examination center. During the in-home interview, a standardized questionnaire was used to collect demographic and health-related information including age, race-ethnicity, sex, level of education, alcohol consumption, reproductive health, and use vitamin supplementation. The response rate for all females during the interview for 2003-2004, 2005-2006, 2007-2008 and 2009-

2010 were 80%, 80.9%, 78.6% and 79.7%, respectively (506-509). During the mobile examination, biochemical parameters were collected along with anthropometric measurements. The response rate during the examination for 2003-2004, 2005-2006, 2007-2008 and 2009-2010 were 76%, 77.8%, 75.5% and 77.4%, respectively (506-509). Subjects with probable hemochromatosis (N = 304) based upon abnormal values of several iron markers (i.e., serum iron > 175 μg/dL, serum ferritin > 200 μg/L, and transferrin saturation > 60%), with reported hepatitis C infection (N = 328) or who reported being pregnant (N = 695) were excluded.

Variable ascertainment and Instrumentation

Predictor Variables In 2003, Cook et al. (50) developed an algorithm to calculate an index of iron repletion, which is given by the equation Fe_{COOK} (mg/kg) = $(-[\log10(R * 1000/F) - 2.8229])/0.1207$, where R = serum transferrin receptor (mg/L) and F = serum ferritin (mcg/L). When the index was developed, serum transferrin receptor concentrations were input into the equation in micrograms per liter. I thus converted milligrams per liter to the required unit by multiplying by 1,000. Serum transferrin receptor is primarily derived from developing red blood cells, reflects the magnitude of erythropoiesis and the body's demand for iron. Ferritin is an intracellular protein whose primary function is iron storage. When measured independently, levels of serum transferrin receptor decrease and levels of ferritin increase with increasing iron intake/absorption. When employing the equation developed by Cook et al., however, the derived value scales proportionately with increasing/decreasing iron repletion, where positive values indicate storage of iron and negative values indicate tissue iron deficiency. In order to develop a more complete understanding of the index, the reader is

encouraged to visit Appendix 1 for a thorough treatment of Fe_{COOK} and a demonstration of the mechanics of the index via eight simulations.

Contributing to the practical value of the equation, iron repletion can be assessed using serum alone, obviating the need for the collection of whole blood. Furthermore, since the function is a ratio of two analytes in serum, it is not adversely affected by hemoconcentration or hemodilution, as hemoglobin or hematocrit would be. Finally, unlike ferritin, the derived value of the function is less altered by chronic inflammation.

During NHANES data collection, serum ferritin was measured using two methods. In 2003, a single-incubation two-site immunoradiometric assay (BioRad Laboratories, Hercules, CA) was used. The assay was discontinued by the manufacturer in early 2004, so ferritin was measured in 2004– 2010 by the Roche Tina-quant ferritin immunoturbidimetric assay on the Hitachi 912 clinical analyzer (Roche Diagnostics, Indianapolis, IN) (510), where latex-bound ferritin antibodies react with the antigen in the same to form an antigen-antibody complex and this was measured turbidimetrically. Because of methodological differences between the BioRad and Roche ferritin assays, concentrations obtained for 2003 samples with the BioRad assay were statistically adjusted in order to be comparable with those obtained for 2004-2010 samples with the Roche assay. This was accomplished before the data release by the National Center for Health Statistics NCHS by applying 3 piecewise linear-regression equations described in detail elsewhere (510).

In accordance with the methods of Cogswell et al. (511), I converted the Roche sTfR concentrations to those equivalent to the Flowers assay used in the development of the body iron model in the original studies. I then applied the same conversion equation

in the current study derived from a previous comparison of the 2 assays used by Cogswell et al. (511): Flowers sTfR = [1.5(Roche sTfR) + 0.35 mg/L]. I used the original Roche ferritin concentrations for the body iron calculation because a previous comparison of the Roche assay with the enzyme-linked immunosorbent assay method used by Skikne et al. (512) and Cook et al. (50) in the calculation of body iron showed no difference between these 2 methods.

From 2003–2010, hemoglobin and hematocrit were measured as part of a complete blood count in the NHANES mobile examination centers with the Beckman CoulterMAXM hematology flow cytometer (Beckman Coulter Inc, Fullerton, CA), where the lytic reagent used for the complete blood count (CBC) parameters prepares blood so that the system can count leukocytes and sense the amount of hemoglobin. The lytic reagent rapidly and simultaneously destroys the erythrocytes and converts a substantial proportion of the hemoglobin to a stable pigment while it leaves leukocyte nuclei intact. The absorbance of the pigment is directly proportional to the hemoglobin concentration of the sample. The accuracy of this method equals that of the hemiglobincyanide method, the reference method of choice for hemoglobinometry recommended by the International Committee for Standardization in Hematology (ICSH).

Hematocrit was computed as the product of red blood cell count (RBC) and mean corpuscular volume (MCV) / 10. Serum iron and unsaturated iron-binding capacity were measured at Collaborative Laboratory Services in Ottumwa, Iowa, by the automated Beckman LX20 (Beckman Coulter, Fullerton, CA). Total iron-binding capacity (TIBC) was calculated indirectly from serum iron and unsaturated iron-binding capacity plus the iron concentration. Transferrin saturation (%) was calculated by dividing the

concentration of serum iron (μmol/L) by the total iron-binding capacity (μmol/L). Dietary and supplemental iron intake was assessed during the in-home interview using a standardized 24-hour dietary recall questionnaire.

Outcome Variables Insulin resistance in the current work was determined among subjects using the homeostasis model assessment (HOMA-IR) developed by Matthews et al. in 1985 to describe glucose and insulin homeostasis via a set of mathematically-derived nonlinear equations (513). The physiological basis for HOMA-IR is encapsulated by the relationship between basal concentrations of glucose and insulin which reflect the action of a negative feedback-loop between hepatic glucose release and pancreatic beta cell activity (513). In this feedback loop, hepatic insulin resistance can be estimated based on reduced insulin secretion by beta cells, which leads to increased hepatic glucose efflux. The resulting increase in plasma glucose stimulates increased secretion of insulin within the portal vein until glucose levels return to normal, thereby completing the feedback loop. The basal plasma insulin levels necessary to maintain normal glucose levels are directly proportional to the degree of insulin resistance (513). Based upon the aforementioned interactions between liver and pancreatic beta cells, Matthews et al. developed an approximating equation for insulin resistance, which is derived from the use of the insulin-glucose product, divided by a constant, and was calculated as

$$HOMA - IR = ((FPI \; x \; FPG))/22.5$$

where *FPI* fasting plasma insulin concentration (mU/L) and *FPG* = fasting plasma glucose (mmol/L). The product of FPG × FPI provides an index of hepatic insulin resistance. The constant of 22.5 is a normalizing factor (i.e., the product of fasting plasma insulin of 5 μU/mL and fasting plasma glucose of 4.5 mmol/L is assumed to be typical of

a typical healthy individual). HOMA-IR has been validated in several study populations to be an accurate, reliable, and non-invasive means with which to determine the presence of insulin resistance in humans.

In the 2003-2004 NHANES sample, fasting plasma insulin concentration (uIU/mL) was measured using the Tosoh AIA-PACK IRI assay (Tosoh Bioscience, Prussia, PA), which is a two-site immunoenzymometric assay performed entirely within the AIA-PACK. For this assay, insulin present in the test sample was bound with a monoclonal antibody which was immobilized on a magnetic solid phase and enzyme-labeled monoclonal antibody within the AIA-PACK. The magnetic beads were washed to remove unbound enzyme-labeled monoclonal antibodies and were then incubated with the fluorogenic substrate, 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labeled monoclonal antibody bound to the beads was directly proportional to the IRI concentration in the test sample. For the years spanning 2005-2010, the Merocodia Insulin ELISA assay (Mercodia Inc, Winston Salem, NC) was used to measure fasting plasma insulin. This assay is a two-site enzyme immunoassay which utilizes the direct sandwich technique where two monoclonal antibodies are directed against separate antigenic determinants of the insulin molecule. Specimen, control, or standard were pipetted into the sample well followed by the addition of peroxidase-conjugated anti-insulin antibodies. Insulin present in the sample binds to anti-insulin antibodies bound to the sample well, while the peroxidase-conjugated anti-insulin antibodies also bind to the insulin at the same time. After washing to remove unbound enzyme-labelled antibodies, a labelled substrate was added which binds to the conjugated antibodies. Acid was then added to the sample well to stop the reaction and the colorimetric endpoint was read on a

microplate spectrophotometer set to the appropriate light wavelength. No conversion was necessary for entry of insulin into the HOMA-insulin resistance equation, as uIU/mL is equivalent to mU/L.

Fasting plasma glucose concentration (mmol/L) was determined by a standard hexokinase method using the Roche Cobas Mira platform for years 2003-2004, the Roche/Hitachi 911 platform for years 2005-2006 and the Roche Modular P chemistry analyzer (Roche Diagnostics, Indianapolis, IN) for years 2007-2010, where the enzyme catalyzes the reaction between glucose and adenosine triphosphate (ATP) to form glucose-6-phosphate (G6P) and adenosine diphosphate (ADP). In the presence of nicotinamide adenine dinucleotide (NAD), G-6-P is oxidized by the enzyme glucose-6-phosphate dehydrogenase (G6PD) to 6-phosphogluconate and reduced nicotinamide adenine dinucleotide (NADH). The increase in NADH concentration is directly proportional to the glucose concentration and can be measured spectrophotometrically at 340 nm.

To determine the presence of non-alcoholic fatty liver disease (NAFLD) among subjects, I used the U.S.-Fatty Liver Index (U.S.-FLI) developed by Ruhl et al. in 2015 which was calculated as

$$\text{U.S.-FLI} = e\hat{}(-0.8073(NHB) + 0.3458(H) + 0.0093(age) + 0.6151(\ln(GGT)) + 0.0249(wc) + 1.1792(\ln(ins)) + 0.8242(\ln(glu)) - 14.7812)/(1 + e\hat{}(-0.8073(NHB) + 0.3458(H) + 0.0093(age) + 0.6151(\ln(GGT)) + 0.0249(wc) + 1.1792(\ln(ins)) + 0.8242(\ln(glu)) - 14.7812)) * 100,$$

where NHB = non-Hispanic black ethnicity (i.e., scored as ‘1’ if the participant is of that ethnicity, ‘0’ otherwise), H = Hispanic ethnicity (i.e., scored as ‘1’ if the participant is of

that ethnicity, ‘0’ otherwise), GGT = gamma-glutamyl transferase (U/L), wc = waist circumference (cm), ins = insulin (uIU/mL), glu = glucose (mg/dL). As is evident in the equation, the U.S.-FLI is an algorithm based upon demographic measures (i.e., ethnicity, age) an anthropomorphic measure (i.e., waist circumference) and analytes obtained from serum (i.e., GGT, insulin, glucose). The U.S.-FLI ranges from 0 to 100 (35) and has been validated with a sensitivity of 62% and specificity of 88% (PPV = 5.2, NPV = 0.43) when using a cut-off of ≥ 30 to define the presence of fatty liver (35).

Ruhl et al. derived the U.S.-FLI using stepwise regression with backward elimination after noting that the development of the fatty liver index (FLI) by Bedogni et al. (30) 1) assumed that insulin was not a widely available measurement in population-based studies, 2) was based upon a homogeneous sample (i.e., the FLI did not account for ethnicity) and 3) did not account for age. As previously mentioned, the prevalence of NAFLD is associated with insulin resistance, varies across ethnicities and increases with age. Thus, an index of fatty liver which incorporates these three factors might be considered more reliable in risk stratification. Moreover, the U.S.-FLI incorporates GGT, which has been shown in previous research (e.g., used alone or with ALT and AST) to be a reliable marker of liver injury. Levels of GGT increase due to excessive alcohol intake, hyperlipidemia, obesity, diabetes or when using certain medications (e.g., aspirin, phenobarbital, phenytoin, steroids, tamoxifen, tetracycline). GGT is expressed within the blood-brain barrier, astrocytes, white blood cells, participates in receptor-ligand interactions at cell membranes and concentration of GGT appears to better differentiate between alcoholic fatty liver disease and non-alcoholic fatty liver disease compared to ALT or AST (514). GGT functions to maintain the available pool of intracellular cysteine

by breaking down extracellular glutathione (GSH). Thus, as levels of intracellular cysteine diminish due to hepatic injury, concentration of GGT increases to convert circulating GSH to its amino acid constituents (i.e., cysteine, glycine, glutamic acid) in order to restore the pool of available cysteine.

From 2003-2006, GGT (U/L) was measured using the Beckman Synchron LX20 (Beckman Coulter, Fullerton, CA). The LX uses an enzymatic rate method to determine the GGT activity in serum or plasma. In the reaction, the GGT catalyzes the transfer of a gamma-glutamyl group from the colorless substrate, gamma-glutamyl- p-nitroaniline, to an acceptor, glycylglycine where the production of a colored product, p-nitroaniline, occurs. The system monitors the rate of change in absorbance at 410 nm over a fixed-time interval. The rate of change in absorbance is directly proportional to the activity of GGT in the sample. From 2007-2010, GGT was measured using the Beckman Synchron LX20 and the Beckman Unicel DxC800 Synchron platform (Beckman Coulter, Fullerton, CA). The DxC800 uses the same chemistry as the LX20 and measurements appear to have been used to cross-validate those obtained from the LX20.

In the current work, the presence of hepatic fibrosis was determined using the method developed by Angulo et al. in 2007, called the NALFD fibrosis score (NFS). The NFS was derived as follows:

$$\text{NFS} = -1.675 + 0.037(\text{age}) + 0.094(\text{BMI}) + 1.13(\text{IFG or T2DM}) + 0.99(AST/ALT) - 0.013(\text{platelets}) - 0.66(\text{albumin}),$$

where BMI = body mass index, IFG = impaired fasting glucose (defined as having a fasting plasma glucose concentration of ≥ 110 mg/dL and T2DM = type 2 diabetes mellitus (i.e., scored as ‘1’ if either condition is present, ‘0’ otherwise)). AST = aspartate

aminotransferase (U/L) and ALT = alanine aminotransferase (U/L). Platelets are entered into the equation in units of cells per liter (i.e., 1000 cells/uL) and albumin is entered into equation as g/dL. According to Augulo et al., advanced fibrosis is defined as having an NFS > 0.676 (515).

During the NHANES mobile examination, unassisted standing height was measured in meters as the maximum value recorded by a fixed stadiometer whose inputs were sent to a computer for reading and storage. Weight (kg) was measured in a standing position by a digital weight scale. Waist circumference was measured in a standing position, above the uppermost lateral border of the right ilium to the nearest 0.1 cm using a soft tape measure. BMI was calculated after obtaining a subject's height and weight via the following standard equation: $(weight\ (kg))/(\ height\ (m)\verb|^|2)$ and categorized in accordance with the WHO BMI classification scheme (i.e., < 18.5 underweight, 18.5 – 24.9 normal weight, 25.0 – 29.9 overweight or > 29.9 obese). After resting quietly in a sitting position for 5 minutes and determining the maximum inflation level after cuff adjustment, three to four systolic and diastolic blood pressure measurements were obtained using a mercury sphygmomanometer in accordance with the protocol of the American Heart Association.

From 2003–2010, platelets were measured as part of a complete blood count in the mobile examination centers with the Beckman CoulterMAXM hematology flow cytometer (Beckman Coulter Inc, Fullerton, CA), where the lytic reagent used for the complete blood count (CBC) parameters modifies the blood in a reaction so that the system can count platelets based upon the principles of light scattering. Platelets were

identified by their size (< 30 FL, low angle light scatter) and refractive index (N = 1.35 to N = 1.40 or high angle light scatter.

The concentration of serum albumin was determined by the Beckman Synchron LX20 using a bichromatic digital endpoint method. Briefly, using this method albumin combines with Bromcresol Purple (BCP) reagent in a reaction to form a complex which absorbs light at 600nm. The system thus monitors the change in absorbance at 600 nm, which is directly proportional to the concentration of albumin in the sample.

From 2003-2006, triglycerides (mg/dL) were measured with the Beckman Synchron LX20 (Beckman Coulter, Fullerton, CA), where a timed-endpoint method was used to determine the concentration of these esters in serum or plasma. Triglycerides in the sample are hydrolyzed to glycerol and free fatty acids by the action of lipase. A sequence of three coupled enzymatic steps using glycerol kinase (GK), glycerophosphate oxidase (GPO), and horseradish peroxidase (HPO) causes the oxidative coupling of 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) with 4-aminoantipyrine to form a red quinoneimine dye. The system monitors the change in absorbance at 520 nm for a fixed-time interval. The change in absorbance is directly proportional to the concentration of triglycerides in the sample. From 2007-2010, trigylcerides were also measured using the Backman Synchron LX20 and the Beckman Unicel DxC800 Synchron platform (Beckman Coulter, Fullerton, CA). The Unicel DxC800 Synchron uses the same chemistry as the LX20 and measurements appear to have been used to cross-validate those obtained from the LX20.

HDL cholesterol (mg/dL) was measured using the direct HDL method, where the lipoprotein is obtained from serum and quantified via an enzymatic reaction on the

Hitachi 912 Analyzer (Roche Diagnostics, Indianapolis, IN). During the assay reaction, apolipoprotein B (apoB) containing lipoproteins in the specimen are reacted against a blocking reagent which renders them non-reactive with a separate enzymatic cholesterol reagent. The apoB containing lipoproteins are thus effectively excluded from the assay and only HDL cholesterol is detected at an absorbance of 600nm.

From 2003-2010, there were three HbA_{1c} laboratory platforms and two laboratories used in NHANES. HbA_{1c} measurement (%) was performed in the first laboratory on the Primus CLC330 platform (Primus Diagnostics, Kansas City, MO) platform from 2003-2004 and in the second laboratory on the Tosoh A1C 2.2 Plus platform (Tosoh Bioscience, Prussia, PA) platform from 2005-2006. From 2007-2010, HbA_{1c} measurements were performed in the second laboratory on the Tosoh A1C G7 platform (Tosoh Bioscience, Prussia, PA). Laboratory method cross-over studies were conducted at the time of each of the laboratory instrument changes. Both laboratories analyzing NHANES HbA_{1c} data from 2003-2010 were standardized by participating in the National Glycohemoglobin Standardization Program (NGSP).

Covariates Age (defined continuously and categorically defined as 12-21 years of age, 22-31 years of age, 32-41 years of age and > 41 years of age), ethnicity (defined as non-Hispanic white, Hispanic and non-Hispanic African American), and education (defined as (1) less than high school diploma, (2) high school diploma/GED, (3) some college or (4) college degree/post-graduate degree) were obtained during the structured in-home interview questionnaire. As mentioned above, anthropomorphic measurements were obtained during the mobile examination and body mass index was calculated using these measurements. Alcohol intake (defined as the number of drinks per day and

categorically defined as 0 drinks/d, 1-3 drinks/d or > 3 drinks/d), smoking status (defined as the number of cigarettes smoked per day and categorically defined as 0 cigarettes/d, 1-5 cigarettes/d or > 5 cigarettes/d) and physical activity (defined as the sum of metabolic equivalent scores (METS) and categorically grouped by METS tertiles) were obtained using the structured in-home interview questionnaire. Data on dietary recall and food frequency was obtained during each participant's mobile examination and again three to ten days later via telephone interview. Age at menarche (defined continuously and categorized as < 12 years of age, 12 years of age or > 12 years of age), oral contraceptive use (dichotomously defined as YES/NO) and parity (defined as the number of children each female has given birth to and categorically defined as 0 children, 1 child, 2-3 children or > 3 children) was obtained during the in-home interview using a structured reproductive history questionnaire. Menopause was defined to align with previous literature (Taylor et al. 2014). Briefly, females aged 18 years of age and older were asked "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries)." Females who answered "no" to this question were subsequently asked "What is the reason that you have not had a period in the past 12 months?" Females were classified as premenopausal if they answered "yes" to the first question or answered "no" to the first question but indicated the reason as pregnancy, breastfeeding, irregular periods or medical conditions/treatments. Females were classified as postmenopausal if they answered "no" to the first question and indicated the reason to be natural menopause or hysterectomy (516).

Summary In the current analysis, I have examined the effect of age, ethnicity, education, body composition, alcohol consumption, smoking history, level of physical activity, dietary and supplemental iron intake, promoters and inhibitors of iron absorption, age at menarche, oral contraceptive use, parity, menopausal status, length of reproductive lifespan and Fe_{COOK} to evaluate their respective impact on metabolic dysfunction.

Variable coding and data analyses

Descriptive Statistics Continuous variables (e.g., age, age at menarche, age at menopause, length of reproductive lifespan, hemoglobin, hematocrit, serum iron, total iron binding capacity, transferrin saturation, dietary iron intake, $Fe_{COOK,}$ fasting plasma insulin, fasting plasma glucose, gamma-glutamyl transferase, triglycerides, waist circumference, systolic blood pressure, diastolic blood pressure, HDL cholesterol, HbA_{1c}) were summarized with standard descriptive statistics including means, medians, standard deviations, and ranges. Categorical variables (e.g., ethnicity, education, BMI, alcohol consumption, smoking history, oral contraceptive use, parity, menopausal status, Fe_{COOK} category, presence/absence of T2DM, presence/absence of metabolic syndrome, U.S.-FLI category, HOMA-IR category) were summarized as frequencies and percentages. Ninety-five percent confidence intervals have been provided for descriptive statistics.

Categorical Outcomes

The presence of Type 2 diabetes mellitus (T2DM) was determined using WHO guidelines and scored as present (i.e., NO = 0, YES = 1) if any of the following criteria are met: fasting glucose ≥ 126 (mg/dL), HbA_{1c} ≥ 6.5 (DCCT%), self-reported diagnosis of T2DM, or self-reported use of medications used to treat T2DM. The presence of metabolic syndrome (metabolic syndrome) was defined according to the definition set forth by ATP III as follows: waist circumference > 35 inches in females, elevated blood pressure (diastolic > 85 mmHg & / systolic > 130 mm Hg in females), HDL cholesterol < 50 mg/dL in females, fasting plasma glucose > 100 mg / dL, and triglycerides > 150 mg/dL and scored as present (i.e., NO = 0, YES = 1) if three or more of the above criteria are met. NAFLD was scored as present (i.e., NO = 0, YES = 1) using the cutoffs developed by Ruhl et al. for the U.S.-FLI, where a score ≥30 denotes the presence of NAFLD (35). NFS was scored as present (i.e., NO = 0, YES = 1) using the cutoff developed by Angulo et al., where a score > 0.676 denotes the presence of advanced fibrosis (515). HOMA-IR was modeled by creating the following categories: < 3: normal insulin resistance, 3-5: moderate insulin resistance, > 5: severe insulin resistance and collapsed into a binary variable and modeled as follows: 0 – 5 normal to moderate insulin resistance, > 5 severe insulin resistance.

If data for the primary predictor variable (i.e., Fe_{COOK}) or any outcome variables with > 2 categories (i.e., NAFLD using U.S.-FLI, insulin resistance using HOMA-IR) were too sparse, data from the first two tertiles/categories was collapsed in order to create a dichotomous variable (e.g., 1st and 2nd vs. 3rd tertile/category for a given variable). Odds of T2DM, metabolic syndrome, NAFLD and/or insulin resistance was determined

between Fe_{COOK} groups using the standard formula $OR = (ai * di))/(bi + di))$, where ai is the number of events for subject i in the highest Fe_{COOK} tertile, bi is the number of non-events for subject i in the highest Fe_{COOK} tertile, ci is the number of events for subject i in the lowest two Fe_{COOK} tertiles, and di is the number of non-events for subject i in the lowest two Fe_{COOK} tertiles.

Categorical outcomes were analyzed using chi-square tests (e.g., one categorical predictor and categorical outcome variable), logistic regression (e.g., one continuous predictor variable and one dichotomous outcome variable), or loglinear models (e.g., > 1 categorical predictor variable and one categorical outcome variable), where appropriate, by applying PROC SURVEY procedures to account for the NHANES study design. Point estimates (i.e., odds ratios) and ninety-five percent confidence intervals have been provided.

Continuous Outcomes

U.S.-FLI and HOMA-IR were also modeled as continuous outcomes to examine the effect of Fe_{COOK} when the latter is treated as both a continuous and categorical predictor. Diagnostics were performed on the data to determine appropriate statistical modeling prior to all analyses. When standard modeling assumptions were upheld for continuous outcomes and when Fe_{COOK} was treated as a continuous predictor, statistically significant associations were determined using linear regression and/or Pearson correlation. When standard modeling assumptions were met for continuous outcomes and when Fe_{COOK} was treated as a categorical predictor, statistically significant differences in outcomes as a function of Fe_{COOK} tertile were evaluated using independent t-tests.

Wilcoxon Rank-Sum tests were applied when standard modeling assumptions were not met.

Power & Sample Size Given the cross-sectional study design of the current secondary analysis, total sample size during continuous NHANES from 2003 to 2010 is immutable. Therefore, I calculated the statistical power to detect each outcome, given the observed sample size. Power estimates were calculated across varying effect sizes, while assuming equal alpha allocation across each of the four metabolic conditions (i.e., $p = 0.05 / 4 = 0.0125$) in accordance with the method proposed by Wittes (517).

Decision Rule The above experiment-wise alpha allocation does not account for multiple testing, thus the Sidak correction (518) was employed using the formula $1 - (1-\alpha)^{1/n}$ where n is the number of independent tests (e.g., in the current proposed study, 10 tests were conducted for each of the four primary hypotheses) and α is the nominal level of statistical significance established in the above power and sample size calculations (i.e., 0.0125) to control for false positive findings. Thus, the level of statistical significance for any finding must fall below $p = 0.00125$. All data management and data analysis programs were written using SAS v9.4 (519).

Statistical Models (in order of each hypothesis listed in the aims section)

Aims 2A1 – 2A6 & Aims 2B1-2B3

a) 2A1 & 2B1: $Fe_{COOK i} = \alpha_i + \beta_{1i}\text{ethnicity}X_{1i} + \varepsilon_i$

b) 2A2: $Fe_{COOK i} = \alpha_i + \beta_{1i}\text{education}X_{1i} + \varepsilon_i$

c) 2A3: $Fe_{COOK i} = \alpha_i + \beta_{1i}\text{BMI}X_{1i} + \varepsilon_i$

d) 2A4: $Fe_{COOK i} = \alpha_i + \beta_{1i}\text{oral contraceptive use}X_{1i} + \varepsilon_i$

e) 2A5: $Fe_{COOK i} = \alpha_i + \beta_{1i}\text{age at menopause}X_{1i} + \varepsilon_i$

f) 2B2: $Fe_{COOK i} = \alpha_i + \beta_{1i}\text{age at menarche}X_{1i} + \varepsilon_i$

g) 2B3: $Fe_{COOK i} = \alpha_i + \beta_{1i}\text{parity}X_{1i} + \varepsilon_i$

where α is the model intercept, β_1 is the beta coefficient associated with predictor variable 1 through n, modeled independently, for subject *i* and ε is the associated error term of the statistical model.

Aim 3A.1 insulin resistance:

a) insulin $resistance_i = \alpha_i + \beta_{1i}Fe_{COOK}X_{1i} + \varepsilon_i$ (Basic Model)

b) insulin $resistance_i = \alpha_i + \beta_{1i}Fe_{COOK}X_{1i} + \beta_{2i}ageX_{2i} + \varepsilon_i$

c) insulin $resistance_i = \alpha_i + \beta_{1i}Fe_{COOK}X_{1i} + \beta_2 ethnicity\ X_{2i} + \varepsilon_i$

d) insulin $resistance_i = \alpha_i + \beta_{1i}Fe_{COOK}X_{1i} + \beta_2 educationX_{2i} + \varepsilon_i$

e) insulin $resistance_i = \alpha_i + \beta_{1i}Fe_{COOK}X_{1i} + \beta 2BMIX_{2i} + \varepsilon_i$

f) insulin $resistance_i = \alpha_i + \beta_{1i}Fe_{COOK}X_{1i} + \beta_2$age at menarche$X_{2i} + \varepsilon_i$

g) insulin $resistance_i = \alpha_i + \beta_{1i}Fe_{COOK}X_{1i} + \beta_2$oral contraceptive use$X_{2i} + \varepsilon_i$

h) insulin $resistance_i = \alpha_i + \beta_{1i}Fe_{COOK}X_{1i} + \beta_2 parityX_{2i} + \varepsilon_i$

i) insulin $resistance_i = \alpha_i + \beta_{1i}Fe_{COOK}X_{1i} + \beta_2$age at menopause$X_{2i} + \varepsilon_i$

j) insulin $resistance_i = \alpha_i + \beta_{1i}Fe_{COOK}X_{1i} + \beta_2$alcohol consumption$X_{2i} + \varepsilon_i$

k) insulin $resistance_i = \alpha_i + \beta_{1i}Fe_{COOK}X_{1i} + \beta_2$smoking history$X_{2i} + \varepsilon_i$

l) insulin $resistance_i = \alpha_i + \beta_{1i}Fe_{COOK}X_{1i} + \beta_2$physical activity$X_{2i} + \varepsilon_i$

m) insulin $resistance_i = \alpha_i + \beta_{1i}Fe_{COOK}X_{1i} + \beta_2$C-reactive protein$X_{2i} + \varepsilon_i$

n) insulin $resistance_i = \alpha_i + Fe_{COOK}$ + age + ethnicity + BMI + alcohol consumption + smoking history + physical activity level + eFe + iFe + C-reactive protein + ε_i, where eFe = enhancers of iron absorption and iFe = inhibitors of iron absorption. (Full Model)

Aim 3A.2 non-alcoholic fatty liver disease:

a) $\text{logit(NAFLD)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \varepsilon_i$ (Basic Model)

b) $\text{logit(NAFLD)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_{2i}\text{ageX}_{2i} + \varepsilon_i$

c) $\text{logit(NAFLD)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{ethnicity X}_{2i} + \varepsilon_i$

d) $\text{logit(NAFLD)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{educationX}_{2i} + \varepsilon_i$

e) $\text{logit(NAFLD)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta\text{2BMIX}_{2i} + \varepsilon_i$

f) $\text{logit(NAFLD)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{age at menarcheX}_{2i} + \varepsilon_i$

g) $\text{logit(NAFLD)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{oral contraceptive useX}_{2i} + \varepsilon_i$

h) $\text{logit(NAFLD)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{parityX}_{2i} + \varepsilon_i$

i) $\text{logit(NAFLD)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{age at menopauseX}_{2i} + \varepsilon_i$

j) $\text{logit(NAFLD)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{alcohol consumptionX}_{2i} + \varepsilon_i$

k) $\text{logit(NAFLD)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{smoking historyX}_{2i} + \varepsilon_i$

l) $\text{logit(NAFLD)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{physical activityX}_{2i} + \varepsilon_i$

m) $\text{logit(NAFLD)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{C-reactive proteinX}_{2i} + \varepsilon_i$

n) $\text{logit(NAFLD)}_i = \alpha_i + \text{Fe}_{\text{COOK}} + \text{age} + \text{ethnicity} + \text{BMI} + \text{alcohol consumption} + \text{smoking history} + \text{physical activity level} + \text{eFe} + \text{iFe} + \text{C-reactive protein} + \varepsilon_{i,}$ where eFe = enhancers of iron absorption and iFe = inhibitors of iron absorption.

(Full Model)

Aim 3A.3 metabolic syndrome:

a) $\text{logit(metabolic syndrome)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}X_{1i} + \varepsilon_i$ (Basic Model)

b) $\text{logit(metabolic syndrome)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}X_{1i} + \beta_{2i}\text{age}X_{2i} + \varepsilon_i$

c) $\text{logit(metabolic syndrome)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}X_{1i} + \beta_2\text{ethnicity } X_{2i} + \varepsilon_i$

d) $\text{logit(metabolic syndrome)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}X_{1i} + \beta_2\text{education}X_{2i} + \varepsilon_i$

e) $\text{logit(metabolic syndrome)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}X_{1i} + \beta 2\text{BMI}X_{2i} + \varepsilon_i$

f) $\text{logit(metabolic syndrome)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}X_{1i} + \beta_2\text{age at menarche}X_{2i} + \varepsilon_i$

g) $\text{logit(metabolic syndrome)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}X_{1i} + \beta_2\text{oral contraceptive use}X_{2i} + \varepsilon_i$

h) $\text{logit(metabolic syndrome)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}X_{1i} + \beta_2\text{parity}X_{2i} + \varepsilon_i$

i) $\text{logit(metabolic syndrome)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}X_{1i} + \beta_2\text{age at menopause}X_{2i} + \varepsilon_i$

j) $\text{logit(metabolic syndrome)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}X_{1i} + \beta_2\text{alcohol consumption}X_{2i} + \varepsilon_i$

k) $\text{logit(metabolic syndrome)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}X_{1i} + \beta_2\text{smoking history}X_{2i} + \varepsilon_{i\backslash}$

l) $\text{logit(metabolic syndrome)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}X_{1i} + \beta_2\text{physical activity}X_{2i} + \varepsilon_i$

m) $\text{logit(metabolic syndrome)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}X_{1i} + \beta_2\text{C-reactive protein}X_{2i} + \varepsilon_i$

n) $\text{logit(metabolic syndrome)}_i = \alpha_i + \text{Fe}_{\text{COOK}}$ + age + ethnicity + BMI + alcohol consumption + smoking history + physical activity level + eFe + iFe + C-reactive protein + $\varepsilon_{i,}$ where eFe = enhancers of iron absorption and iFe = inhibitors of iron absorption. (Full Model)

Aim 3A.4 Type 2 diabetes mellitus:

a) $\text{logit(T2DM)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \varepsilon_i$ (Basic Model)

b) $\text{logit(T2DM)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_{2i}\text{ageX}_{2i} + \varepsilon_i$

c) $\text{logit(T2DM)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{ethnicity X}_{2i} + \varepsilon_i$

d) $\text{logit(T2DM)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{educationX}_{2i} + \varepsilon_i$

e) $\text{logit(T2DM)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta\text{2BMIX}_{2i} + \varepsilon_i$

f) $\text{logit(T2DM)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{age at menarcheX}_{2i} + \varepsilon_i$

g) $\text{logit(T2DM)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{oral contraceptive useX}_{2i} + \varepsilon_i$

h) $\text{logit(T2DM)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{parityX}_{2i} + \varepsilon_i$

i) $\text{logit(T2DM)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{age at menopauseX}_{2i} + \varepsilon_i$

j) $\text{logit(T2DM)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{alcohol consumptionX}_{2i} + \varepsilon_i$

k) $\text{logit(T2DM)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{smoking historyX}_{2i} + \varepsilon_i$

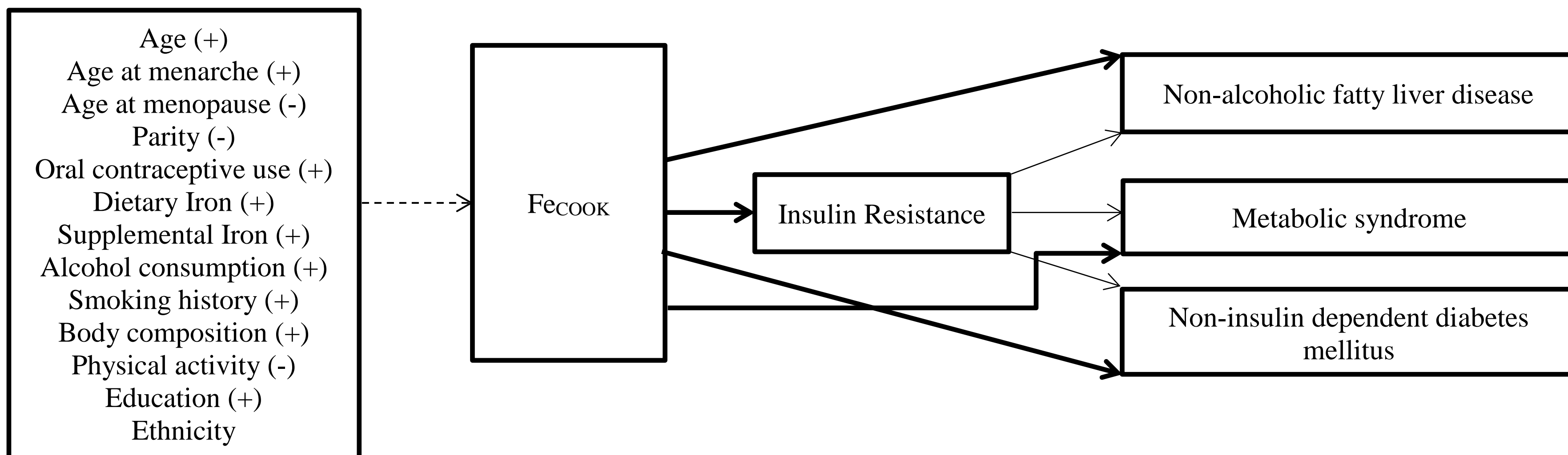
l) $\text{logit(T2DM)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{physical activityX}_{2i} + \varepsilon_i$

m) $\text{logit(T2DM)}_i = \alpha_i + \beta_{1i}\text{Fe}_{\text{COOK}}\text{X}_{1i} + \beta_2\text{C-reactive proteinX}_{2i} + \varepsilon_i$

n) $\text{logit(T2DM)}_i = \alpha_i + \text{Fe}_{\text{COOK}}$ + age + ethnicity + BMI + alcohol consumption + smoking history + physical activity level + eFe + iFe + C-reactive protein + $\varepsilon_{i,}$ where eFe = enhancers of iron absorption and iFe = inhibitors of iron absorption.

(Full Model)

In terms of a conceptual model, the above associations and statistical models can be visualized as follows:



Dashed arrow indicates hypothesized mediation. Bolded arrows identify hypotheses tested in proposed statistical models.

CHAPTER 6: RESULTS

Data from 14,895 females aged 12 years and older during the years 2003-2010 were available for analysis in current study. After excluding females on the basis of probable hemochromatosis (i.e., serum iron > 175 μg/dL, serum ferritin > 200 μg/L, and transferrin saturation > 60%) (N = 304), who reported hepatitis C infection (N = 328) or who reported being pregnant (N = 695), a total of 13,568 remained eligible for inclusion from the initial pool of participants. Of these eligible females, 7,461 had complete data that could be used for the calculation of iron repletion (i.e., Fe_{COOK}) (Figure 1).

Demographics The mean age of females in this sample was 27.21 years (sd = 11.64, range: 12 – 49). White females comprised the largest proportion of the sample population (42.65%, N = 3182), followed by Hispanic (32.30%, N = 2,432) and black females (24.76%, N = 1,846) (Table 1). Roughly half of the sample population (52.97%) reported having less than a high school education, 13.22% reported having a high school diploma/GED equivalent, and 13.34% reported having a college or post-graduate degree. Four females refused to provide information regarding education (Table 1). When education was stratified by ethnicity, a greater percentage of white females reported having a college or post-graduate degree compared to black or Hispanic females (68.1% vs 17.6% and 14.3%, respectively).

The average height in the sample population was 161.48 cm (sd = 7.03 cm, range: 132.9 – 194.2 cm). White females tended to be slightly taller than Hispanic and black females (ẍ = 163.48 cm, sd = 6.68 cm vs 158.03 cm, sd = 6.37 cm and 162.60 cm, sd = 6.75 cm, respectively). The average weight for females in the current sample was 71.01 kg (sd = 20.97 kg: range 24.0-211.7 kg). Black females tended to weigh more than white or Hispanic females (ẍ = 76.37 kg, sd = 24.19 kg vs 70.57 kg, sd = 20.42 kg and 67.53 kg, sd; = 18.05 kg, respectively). The average waist circumference for participants was 89.33 cm (sd = 16.85, range: 55.0 - 176.7 cm). Black females tended to have larger waist circumferences than white or Hispanic females (ẍ = 90.74 cm, sd = 19.20 cm vs 88.67 cm, sd = 16.67 cm and 88.14 cm, sd = 15.05 cm, respectively. The mean BMI for females in the sample population was 27.14 (sd = 7.47, range: 13.59 – 84.87). Most females had a BMI between 18.5 and 24.9 (41.21%), followed by 29.45% having a BMI > 29.9, 23.88% having a BMI between 25.0 and 29.9 and 5.46% having a BMI < 18.5 (Table 1). On average, white females had a lower BMI (ẍ = 26.34, sd = 7.20) than Hispanic females (ẍ = 26.97, sd = 6.70) and black females (ẍ = 28.77, sd = 8.56). Moreover, when collapsing the lowest two WHO BMI categories and collapsing the highest two WHO BMI categories to create a dichotomous BMI variable (e.g., underweight to normal weight vs. overweight to obese), a greater proportion of white females were categorized in the former group compared to the latter (53.12% vs. 46.88%). This pattern reversed for Hispanic females (44.02% vs. 55.98%) and was more pronounced for black females (39.04% vs. 60.96%). When BMI was further stratified by ethnicity, more white females had a BMI < 18.5 or between 18.5 and 24.9 than Hispanic or black females (47.64% vs. 29.53% and 22.83%; 48.67% vs. 30.96% and 20.37%, respectively). White and Hispanic

females had similar proportions of BMIs between 25.0 and 29.9 (38.84% and 37.25%, respectively), while the proportion of black females in this BMI range was smaller (23.91%). More white females than Hispanic or black females had a BMI > 29.9 (36.42% vs. 31.86% and 31.72%, respectively). As mentioned, however, white females tended to have lower BMIs than Hispanic or black females.

Among females who reported drinking alcoholic beverages, the average number of drinks consumed per day was 0.97 (sd = 1.75). The greatest proportion of females (62.14%) reported that they did not drink alcoholic beverages, while 30.29% reporting consuming between 1 to 3 drinks per day and 7.57% reported consuming greater than 3 alcoholic beverages a day (Table 1). When alcohol consumption was stratified by ethnicity, a greater proportion of white and Hispanic than black females (35.47% and 36.07% vs. 28.46%), reported abstinence from drinking among those in this respondent category. Among those who did report consuming alcohol, a greater proportion of white females (54.46%) reported drinking than Hispanic (26.84%) or black females (18.70%). When categorized further (i.e., 1 – 3 drinks/d vs > 3 drinks/d), a greater proportion of white females (54.09%) than Hispanic (25.23%) or black females (20.67%) reported alcohol consumption in this respondent category. Similar patterns were observed for females reporting to drink greater than 3 alcoholic beverages a day, where a greater proportion of white females than Hispanic or black females reported alcohol consumption in this respondent category (55.93% vs 33.27% and 10.80%, respectively).

Among females who reported smoking cigarettes, the average number of cigarettes smoked per day was 1.92 (sd = 5.87). Similar to the overall pattern observed for alcohol consumption, however, 82.29% of the sample reported not smoking. The

remaining 6.78% of females reported smoking 1 – 5 cigarettes per day and 10.93% of females reported smoking > 5 cigarettes per day. When smoking was stratified by ethnicity, a greater proportion of white and Hispanic than black females (38.51% and 35.39% vs. 26.10%), reported not smoking among respondents in this category. Among those who smoked, a greater proportion of white females (61.92%) reported smoking than Hispanic (19.53%) or black females (18.55%). When categorized further (i.e., 1 – 5 vs > 5 cigarettes/d), a greater proportion of white females reported smoking 1 – 5 cigarettes per day compared to Hispanic or black females (41.70% compared to 36.56% and 21.74%, respectively). Similar patterns were observed for females in the highest smoking category, where a greater proportion of white females (74.48%) reported smoking > 5 cigarettes per day compared to Hispanic females (8.96%) and black females (16.56%).

The average METS score in the current sample was 9.97 (sd = 9.24). On average, white females were more active than black and Hispanic females: ẍ = 10.17 (sd = 9.15) for white females, ẍ = 9.73 (sd = 8.48) for black females and ẍ = 9.29 (sd = 8.49) for Hispanic females. When METS scores were grouped into tertiles, a greater proportion of white females were in the highest tertile of METS scores compared to black and Hispanic females (38.30%, compared to 30.40% and 26.69%, respectively, $P < 0.0001$).

Females in the current sample reported a mean dietary intake of 13.93 mg/day (sd = 11.69 mg/day). When stratified by ethnicity, white females reported higher intakes of dietary iron than Hispanic and black females: ẍ = 14.06 (sd = 10.35), ẍ = 13.88 (sd = 13.51) and ẍ = 13.28 (sd = 10.03), respectively), which was not statistically significant ($P = 0.35$). A moderate proportion of females in the current sample reported using iron

supplements (35.74% (95%CI: 32.23-39.25). When iron supplement use was stratified by ethnicity, a greater proportion of black females compared to Hispanic and white females reported using iron supplements (40.46% compared to 38.09% and 34.42%, $P = 0.17$). In terms of dietary composition (i.e., USDA food groups), most of the total iron intake among all females was obtained from grains and meats: 57.16% and 16.98%, respectively for white females, 58.03% and 17.87% for Hispanic females and 52.66% and 22.98% for black females. Taken together, black females obtained more of their total daily iron intake from grains and meats compared to their white and Hispanic counterparts (Figure 2).

For convenience, dietary intakes, supplement intakes and dietary composition of foodstuffs considered as covariates in the current work are summarized below in terms of their effect on iron absorption (i.e., enhancers and inhibitors of iron absorption).

Promoters of iron absorption The average intake of saturated fats among females in the current sample was 22.56 g/d (sd = 19.06 g/d). White females reported higher intakes of saturated fats than black and Hispanic females: 23.09 g/d (sd = 16.06 g/d), 22.63 g/d (sd = 16.09 g/d) and 20.11 g/d (sd = 17.12 g/d), respectively, a small fraction of which was obtained from supplements containing saturated fats. Only 3.04% of white females reported taking supplements containing saturated fats, followed by Hispanic females and black females (2.39% and 1.54%, respectively, $P = 0.20$). Overall, most saturated fats were obtained from milk and milk products, meat, poultry and fish and grains. The distribution across ethnicities, however, differed. For instance, among white females, the dietary composition of saturated fats obtained from each of the aforementioned USDA food groups was 27.84%, 21.46% and 28.58%. Among Hispanic

females, the dietary composition of saturated fats obtained from each food group was 25.88%, 23.18% and 29.49%. Among black females, the dietary composition of saturated fats obtained from each food group was 18.76%, 31.06% and 27.76%. Taken together, black females obtained less saturated fats from milk and milk products and more saturated fats from meats compared to their white and Hispanic counterparts (Figure 3).

The average intake of carbohydrates/sugars among females in the current sample was 228.42 g/day (sd = 124.63). Overall, black females reported higher intakes of carbohydrates/sugars than Hispanic and white females: 234.11 g/d (sd = 143.04 g/d), 229.99 g/d (sd = 154.44 g/d) and 227.01 g/d (sd = 106.70 g/d), respectively, a small fraction of which was obtained from supplements containing carbohydrates/sugars. Stratified by ethnicity, 16.67% of white females reported taking supplements containing carbohydrates/sugars, followed by black females and Hispanic females (10.25% and 8.82%, respectively, $P = 0.002$). Overall, most carbohydrates/sugars were obtained from grains and sugar/sweeteners/beverages. As with saturated fats, the dietary composition/distribution of carbohydrates/sugar dietary differed across ethnicities. For instance, among white females, the dietary composition of carbohydrates/sugars obtained from each of the aforementioned USDA food groups was 42.94% and 24.78%. Among Hispanic females, the dietary composition of carbohydrates/sugars obtained from each food group was 44.14% and 24.20%. Among black females, the dietary composition of carbohydrates/sugars obtained from each food group was 37.50% and 31.59%. Taken together, black females obtained fewer carbohydrates/sugars from grains and more carbohydrates/sugars from sugar/sweeteners/beverages compared to their white and Hispanic counterparts (Figure 4).

The average vitamin C intake among females in the current sample was 79.07 mg/day (sd = 126.84 mg/d). Hispanic females reported higher intakes of vitamin C than black and white females: 95.99 mg/d (sd = 118.23 mg/d), 91.20 mg/d (sd = 124.62 mg/d) and 73.07 mg/d (sd = 102.58 mg/d), respectively, a substantial fraction of which was obtained from supplements containing vitamin C. When stratified by ethnicity, 48.43% of Hispanic females reported taking supplements containing vitamin C, followed by black and white females (46.17% and 44.42%, respectively, $P = 0.39$). Overall, most vitamin C was obtained from fruits, vegetables and sugar/sweeteners/beverages. The composition/distribution across ethnicities differed for vitamin C as well. For instance, among white females, the dietary composition of vitamin C obtained from each of the aforementioned USDA food groups was 39.82%, 26.06% and 15.95%. Among Hispanic females, the dietary composition of saturated fats obtained from each food group was 45.80%, 19.11% and 19.96%. Among black females, the dietary composition of vitamin C obtained from each food group was 43.23%, 15.36% and 32.43%. Taken together, black females obtained more vitamin C from sugar/sweeteners/beverages than their white and Hispanic counterparts and white females obtained less vitamin C from fruits and more vitamin C from vegetables from their Hispanic and black counterparts (Figure 5).

Inhibitors of iron absorption The average intake of fiber among females in the current sample was 14 g/d (sd = 13.74 g/d). Hispanic females reported higher intakes of fiber than white and black females: 15.04 g/d (sd = 12.69 g/d), 14.18 g/d (sd = 13.09 g/d) and 11.79 g/d (sd = 9.75 g/d), respectively, a small fraction of which was obtained from supplements containing fiber. Only 3.09% of white females reported taking supplements containing fiber, followed by black females and Hispanic females (2.37% and 1.84%,

respectively, $P = 0.45$). Overall, most fiber was obtained from grains, fruits and vegetables. The distribution across ethnicities, however, differed. For instance, among white females, the dietary composition of fiber obtained from each of the aforementioned USDA food groups was 46.28%, 11.45% and 20.92%. Among Hispanic females, the dietary composition of fiber obtained from each food group was 46.81%, 15.18% and 15.96%. Among black females, the dietary composition of fiber obtained from each food group was 43.95%, 10.76% and 23.75%. Taken together, black females obtained less fiber from grains and more fiber from vegetables compared to their white and Hispanic counterparts (Figure 6).

The average intake of calcium among females in the current sample was 860.46 mg/d (sd = 778.17 mg/d). White females reported higher intakes of calcium than Hispanic and black females: 890.88 mg/d (sd = 675.16 mg/d), 832.74 mg/d (sd = 767.09 mg/d) and 729.08 mg/d (sd = 497.14 mg/d), respectively, a substantial fraction of which was obtained from supplements containing calcium. When stratified by ethnicity, 57.04% of Hispanic females reported taking supplements containing calcium, followed by white females and black females (52.53% and 49.18%, respectively, $P = 0.32$). Overall, most calcium was obtained from milk and milk products, grains, sugar/sweeteners/beverages, meat and fruits. The composition/distribution of calcium differed across ethnicities as well. For instance, among white females, the dietary composition of calcium obtained from each of the aforementioned USDA food groups was 41.76%, 27.73%, 11.42%, 6.48% and 3.61%. Among Hispanic females, the dietary composition of calcium obtained from each food group was 37.21%, 28.30%, 12.13%, 6.48% and 7.13%. Among black females, the dietary composition of calcium obtained from each food group was 28.66%,

30.65%, 12.81%, 10.84% and 7.07%. Taken together, black females obtained less calcium from milk and milk products and more calcium from meats compared to their white and Hispanic counterparts (Figure 7).

The average intake of magnesium among females in the current sample was 247.79 mg/d (sd = 216.12 mg/d). White females reported higher intakes of magnesium than Hispanic and black females: 254.67 mg/d (sd = 203.46 mg/d), 246.08 mg/d (sd = 165.71 mg/d) and 212.61 mg/d (sd = 117.10 mg/d), respectively, a moderate fraction of which was obtained from supplements containing magnesium. When stratified by ethnicity, 38.92% of Hispanic females reported taking supplements containing magnesium, followed by white and black females (37.35% and 34.37%, respectively, P = 0.71). Overall, most magnesium was obtained from grains, meats and milk/milk products. The distribution across ethnicities, however, differed. For instance, among white females, the dietary composition of magnesium obtained from each of the aforementioned USDA food groups was 30.27%, 13.18% and 13.18%. Among Hispanic females, the dietary composition of magnesium obtained from each food group was 34.35%, 15.15% and 10.57%. Among black females, the dietary composition of magnesium obtained from each food group was 29.54%, 18.86% and 8.11%. Taken together, white females obtained most of their magnesium from milk/milk products, Hispanic females obtained most of their magnesium from grains and black females obtained most of their magnesium from meat (Figure 8).

The average vitamin D intake among females in the current sample was 4.35 µg/d (sd = 5.59 µg/d). White females reported higher intakes of vitamin D than Hispanic and black females: 4.50 µg/d (sd = 5.11 µg/d), 4.47 µg/d (sd = 5.29 µg/d) and 3.44 µg/d (sd =

2.89 μg/d), respectively, a moderate fraction of which was obtained from supplements containing vitamin D. When stratified by ethnicity, 48.92% of black females reported taking supplements containing vitamin D, followed by Hispanic and white females (48.13% and 43.18%, respectively, $P = 0.09$). Overall, most vitamin D was obtained from milk/milk products, meat, grains and eggs. As with the above patterns, the composition/distribution of vitamin D in the diet differed across ethnicities as well. For instance, among white females, the dietary composition of vitamin D obtained from each of the aforementioned USDA food groups was 51.52%, 17.98%, 14.18% and 6.16%. Among Hispanic females, the dietary composition of vitamin D obtained from each food group was 47.43%, 18.46%, 11.95% and 9.39%. Among black females, the dietary composition of vitamin D obtained from each food group was 37.48%, 26.19%, 16.23% and 8.31%. Taken together, white females obtained most of their vitamin D from milk/milk products, Hispanic females obtained most of their vitamin D from eggs and black females obtained most of their vitamin D from meat and grains (Figure 9).

The average vitamin E intake among females in the current sample was 6.51 mg/d (sd = 8.96 mg/d). White females reported higher intakes of vitamin E than black and Hispanic females: 6.74 mg/d (sd = 8.27 mg/d), 5.99 mg/d (sd = 4.05 mg/d) and 5.89 mg/d (sd = 5.97 mg/d). Overall, most vitamin E was obtained from grains, vegetables, meat, legumes/seeds and milk/milk products. The distribution across ethnicities, however, differed. For instance, among white females, the dietary composition of vitamin E obtained from each of the aforementioned USDA food groups was 32.67%, 15.97%, 11.97%, 12.82% and 6.19%. Among Hispanic females, the dietary composition of vitamin E obtained from each food group was 34.10%, 15.81%, 14.45%, 8.85% and

3.43%. Among black females, the dietary composition of vitamin E obtained from each food group was 31.10%, 20.69%, 16.81%, 8.13% and 3.05%. Taken together, white females obtained most of their vitamin E from milk/milk products, legumes, nuts and seeds, Hispanic females obtained most of their vitamin E from grains and black females obtained most of their vitamin E from meat and vegetables (Figure 10).

The average zinc intake among females in the current sample was 10.17 mg/d (sd = 8.74 mg/d). White females reported higher intakes of zinc than Hispanic and black females: 10.32 mg/d (sd = 6.17 mg/d), 9.96 mg/d (sd = 12.41 mg/d) and 9.62 mg/d (sd = 8.84 mg/d), respectively, a moderate fraction of which was obtained from supplements containing zinc. When stratified by ethnicity, 40.12% of Hispanic and black females reported taking supplements containing zinc, followed by white females (37.56%, P = 0.59). Overall, most zinc was obtained from meat, grains and milk/milk products. The distribution/composition of zinc, however, differed across ethnicities. For instance, among white females, the dietary composition of zinc obtained from each of the aforementioned USDA food groups was 32.14%, 33.87%, and 15.90%. Among Hispanic females, the dietary composition of zinc obtained from each food group was 36.14%, 33.75% and 13.07%. Among black females, the dietary composition of zinc obtained from each food group was 43.53%, 29.16% and 8.81%. Taken together, white females obtained most of their zinc from milk/milk products and grains compared to their Hispanic and black counterparts who obtained most of their zinc from meat (Figure 11).

Reproductive Lifespan The mean age of menarche among the current sample population was 12.36 years (sd = 1.61, range: 7 – 19) (Table 1). Menarche occurred slightly earlier for Hispanic and black females ($\ddot{x}$ = 12.33) compared to white females ($\ddot{x}$

= 12.62). When age at menarche was categorized as < 12 years of age, 12 years old, or > 12 years of age, 27.75% of the sample population reported experiencing menarche at < 12 years of age, 28.33% reported experiencing menarche at age 12 and 43.92% reported experiencing menarche > 12 years of age (Table 1). When stratified by ethnicity, 35.08% of white females, 34.91% of Hispanic females and 30.01% of black females reported experiencing menarche at < 12 years of age in this respondent category. Among those who reported experiencing menarche at 12 years of age, a greater proportion of white females (42.36%) than Hispanic or black females (33.17% and 24.47%, respectively) reported experiencing menarche in this respondent category. Similar patterns were observed among those reporting experiencing menarche > 12 years of age, where a greater proportion of white females than Hispanic or black females (48.99% vs. 30.42% and 20.59%, respectively) reported experiencing menarche in this group of respondents.

Roughly half of the sample population reported having ever used oral contraceptives (53.11%) (Table 2) and the mean age of oral contraceptive users was 33.07 (sd = 9.94). When stratified by ethnicity, more white females than Hispanic or black females reported ever having used oral contraceptive (53.50% vs. 25.14% and 21.36%, respectively). Most of the females in the current sample were nulliparous (49.51%). Among those females reporting being pregnant at least one time, the average number of pregnancies was 2.98 (sd = 1.93, range: 1 – 34). When the response was categorized as 1 child, 2 to 3 children, or > 3 children, 10.02% of females reported having a single child, 24.92% reported having between 2 to 3 children, and 15.55% reported having > 3 children. When this variable was stratified by ethnicity, a greater proportion of white females than Hispanic or black females reported having one child in this

respondent category (48.85% vs. 30.53% and 20.61%, respectively). Similarly, a greater proportion of white females than Hispanic or black females reported having 2 to 3 children in this respondent category (47.05% vs. 31.51% and 21.44%, respectively). A similar pattern was observed in the final respondent category, where a greater proportion of white females reported having > 3 children than Hispanic or black females (39.67% vs. 34.94% and 25.39%, respectively). The mean age of menopause in the sample population was 43.94 (sd = 4.80, range: 20 – 49) (Table 2). This variable was then categorized in accordance with common definitions for premature menopause (i.e., < 40 years of age), early menopause (i.e., between 40 and 45 years of age) and natural menopause (> 45 years of age) (Shuster et al.). Grouped in this manner, 16.99%, 35.38% and 47.63% of females in the sample experienced premature menopause, early menopause and natural menopause, respectively (Table 2). When stratified by ethnicity, a greater proportion of white females than Hispanic or black females reported experiencing premature menopause (68.85% compared to 16.39% and 14.75%, respectively). Similarly, a greater proportion of white females than Hispanic or black females reported experiencing early menopause (51.97% compared to 22.05 and 25.98%, respectively). The same pattern held for natural menopause, where a greater proportion of white females reported experiencing menopause naturally (53.80%), followed by black females (25.15%) and Hispanic females (21.05%). The average length of the reproductive lifespan among females in this sample was 27.55 years (sd=51.10). When stratified by ethnicity, black females had the longest reproductive lifespan (36.94 years, sd = 10.17), followed by Hispanic females (26.11 years, sd = 5.86) and white females (24.10 years, sd = 7.28) (Table 2).

Iron parameters Mean hemoglobin (Hb) concentration among the sample population was 13.44 g/dL (sd = 2.96 g/dL) (Table 3). When stratified by ethnicity, white females had the highest Hb concentration (ẍ = 13.64 g/dL, sd = 2.19 g/dL), followed by Hispanic females (ẍ = 13.26 g/dL, sd = 1.90 g/dL) and black females (ẍ = 12.61 g/dL, sd = 1.23 g/dL). Ethnicity explained ~ 10% of the variation in Hb concentration in the current sample (r-squared = 0.09, $P < 0.0001$). Mean hematocrit (Hct) among the sample population was 39.39% (sd = 9.09%) (Table 2). When stratified by ethnicity, white females had the highest Hct (ẍ = 39.83%, sd = 7.36%), followed by Hispanic females (ẍ = 38.89%, sd = 5.99%) and black females (ẍ = 37.62%, sd = 4.35%). Ethnicity explained ~ 6% of the variation in Hct concentration in the current sample (r-squared = 0.06, $P < 0.0001$). Mean serum iron concentration (μg/dL) among the sample population was 77.42 μg/dL (sd = 48.74 μg/dL) (Table 2). When stratified by ethnicity, white females had the highest serum iron concentration (ẍ = 80.32 μg/dL, sd = 45.25 μg/dL), followed by Hispanic females (ẍ = 73.58 μg/dL, sd = 42.65 μg/dL) and black females (ẍ = 66.61 μg/dL, sd = 25.65 μg/dL). Ethnicity explained ~ 2% of the variation in serum iron concentration in the current sample (r-squared = 0.02, $P < 0.0001$). Mean total iron binding capacity (TIBC) among the sample population was 365.02 μg/dL (sd = 102.28 μg/dL) (Table 2). When stratified by ethnicity, Hispanic females had the highest TIBC concentration (ẍ = 371.13 μg/dL, sd = 76.10 μg/dL), followed by white females (ẍ = 364.26 μg/dL, sd = 83.02 μg/dL) and black females (ẍ = 362.60 μg/dL, sd = 58.86 μg/dL). Ethnicity explained < 1% of the variation in TIBC concentration in the current sample (r-squared = 0.002, $P = 0.52$). Mean transferrin saturation (TSAT) among the sample population was 21.84% (sd = 13.68%) (Table 2). When stratified by ethnicity,

white females had the highest TSAT (ẍ = 22.64%, sd = 13.29%) followed by Hispanic females (ẍ = 20.45%, sd = 13.63%) and black females (ẍ = 19.13%, sd = 8.73%). Ethnicity explained ~ 1% of the variation in TSAT concentration in the current sample (r-squared = 0.01, $P = 0.0007$). Mean serum transferrin receptor (sTfR) concentration (mg/L) among the sample population was 3.59 mg/L (sd = 2.47 mg/L) (Table 2). When stratified by ethnicity, black females had the highest sTfR concentration (ẍ = 4.48 mg/L, sd = 3.23 mg/L), followed by Hispanic females (ẍ = 3.63 mg/L, sd = 2.37 mg/L) and white females (ẍ = 3.41 mg/L, sd = 1.97 mg/L). Ethnicity explained ~ 4% of the variation in sTfR concentration in the current sample (r-squared = 0.04, $P = 0.002$). Mean ferritin concentration (µg/L) among the sample population was 47.94 µg/L (sd = 47.47 µg/L) (Table 2). When stratified by ethnicity, white females had the highest ferritin concentration (ẍ = 49.22 µg/L, sd = 39.03 µg/L), followed by black females (ẍ = 46.95 µg/L, sd = 41.81 µg/L) and Hispanic females (ẍ = 43.07 µg/L, sd = 59.51 µg/L). Ethnicity explained < 1% of the variation in serum ferritin concentration in the current sample (r-squared = 0.004, $P = 0.08$).

Total Body Iron and Demographics Mean iron stores via Cook's iron repletion index (i.e., Fe_{COOK}) in the sample were 6.98 mg/kg (sd = 4.82 mg/kg) (Table 3). White females were more iron replete (Fe_{COOK} ẍ = 7.27 mg/kg, sd = 4.28 mg/kg), followed by Hispanic females (Fe_{COOK} ẍ = 6.52 mg/kg, sd = 6.81 mg/kg), and black females (Fe_{COOK} ẍ = 5.95 mg/kg, sd = 4.24 mg/kg) ($P < 0.0001$, Figure 12). Ethnicity explained ~ 2% of the variation in Fe_{COOK} concentration in the current sample (r-squared = 0.02, $P < 0.0001$). Fe_{COOK} was linearly related to age, where Fe_{COOK} increased 0.03 mg/kg with each increasing year of age ($P < 0.0001$). The same trend was observed when stratified

by ethnicity, where Fe_{COOK} increased 0.03 mg/kg in white females ($P < 0.0001$), 0.02 mg/kg for Hispanic females ($P = 0.03$) and 0.02 mg/kg in black females ($P = 0.06$) with each increasing year of age. Similar patterns were observed when age was treated as a categorical variable, where Fe_{COOK} increased across age categories. Fe_{COOK} increased from 6.32 mg/kg among those in the 12-21 year-old age group to 7.21 mg/kg among those in the 22-31 year-old age group, decreased slightly among those in the 32-41 year-old age group to 7.10 mg/kg and increased again among those in the > 41 year-old age group (Fe_{COOK} $\ddot{x} = 7.32$ mg/kg) ($P < 0.0001$, Figure 13). The same trend appeared when stratified by ethnicity, where Fe_{COOK} increased across the first two age categories (i.e., 12-21y and 22-31y), decreased in the third age category (i.e., 32-41y) and increased again in the highest age category (> 41 years of age) for white and Hispanic females and increased monotonically across age groups in black females (Figure 14).

Mean Fe_{COOK} increased with increasing education, where females in the highest category of education had higher Fe_{COOK} ($\ddot{x} = 6.48$ among those with less than a high school education, $\ddot{x} = 7.08$ among those with a high school education/GED, $\ddot{x} = 7.21$ among those with some college and $\ddot{x} = 7.38$ among those with a college degree/post-graduate education) ($P < 0.0001$, Figure 15). This trend diverged slightly when stratifying by ethnicity (Figure 16). Fe_{COOK} increased across the lowest two categories of education, decreased slightly in the third category of educational attainment and increased again in the highest category for white females ($\ddot{x} = 6.73$, $\ddot{x} = 7.48$, $\ddot{x} = 7.43$, $\ddot{x} = 7.57$, $P < 0.0001$). For Hispanic females Fe_{COOK} increased across the first three categories of educational attainment and decreased in the highest category ($\ddot{x} = 6.23$, $\ddot{x} = 6.72$, $\ddot{x} = 6.96$, $\ddot{x} = 6.76$, $P = 0.05$). For black females, a saw-tooth pattern emerged where Fe_{COOK}

decreased from the lowest category of educational attainment to the second category, increased significantly among females in the third category and decreased again among females in the highest category of educational attainment (ẍ = 5.88, ẍ = 5.45, ẍ = 6.35, ẍ = 5.86, $P = 0.05$).

Fe_{COOK} was inversely associated with height (b1 = -0.03, t = -1.23, $P = 0.22$). When stratified by ethnicity, this effect was more pronounced in white and black females compared to Hispanic females (b1 = -0.06 mg/kg, t = -2.16, $P = 0.03$ for white females, b1 = -0.05 mg/kg, t = -1.06, $P = 0.29$ for black females and b1 = -0.03, t = -0.86, $P = 0.39$ for Hispanic females). When height and Fe_{COOK} were categorized according to tertiles, the same pattern emerged where increasing height was associated with decreasing Fe_{COOK} ($P_{trend} = 0.01$) and the trend persisted after stratifying by ethnicity, where taller females within each ethnicity had lower Fe_{COOK} than shorter females.

Fe_{COOK} was linearly associated with weight and waist circumference, where Fe_{COOK} increased 0.009 mg/kg in with each increasing kilogram of weight ($P < 0.0001$) and 0.01 mg/kg for each increasing centimeter of waist circumference ($P < 0.0001$). Fe_{COOK} was linearly related to BMI, where Fe_{COOK} increased 0.01 mg/kg with each increasing unit of BMI ($P = 0.05$). When BMI was categorized in accordance with the risk-based strata developed by the WHO, a similar pattern was observed. However, the linear trend was no longer statistically significant ($P = 0.19$, Figure 17).

Fe_{COOK} was linearly related to alcohol consumption, where Fe_{COOK} increased 0.23 mg/kg with each increasing unit of self-reported daily alcohol intake ($P < 0.0001$). The same trend was observed after stratifying by ethnicity, though the magnitude of the association was greater for white females compared to black and Hispanic females (b1 =

0.23 mg/kg, $P < 0.0001$) vs 0.20 mg/kg ($P = 0.01$) and 0.13mg/kg ($P = 0.02$), respectively, for each unit of increasing alcohol intake. When alcohol intake was categorized into three groups (i.e., 0 drinks/d, 1-3 drinks/d or > 3 drinks/d), a similar pattern emerged where Fe_{COOK} increased 0.68 mg/kg with each increasing category ($P <$ 0.0001, Figure 18). Again, the same trend was observed after stratifying by ethnicity, where the magnitude of the association was greater for white females compared to black and Hispanic females (b1 = 0.68 mg/kg, $P < 0.0001$) vs 0.37 mg/kg ($P = 0.08$) and 0.40 mg/kg ($P = 0.01$), respectively, for each increasing category of alcohol intake (Figure 19).

Fe_{COOK} was positively associated with smoking, where Fe_{COOK} increased 0.05 mg/kg with each increasing cigarette ($P < 0.0001$). The same trend was observed after stratifying by ethnicity, though the magnitude of the association was greater for white females compared to black and Hispanic females (b1 = 0.04 mg/kg, $P < 0.0001$) vs 0.01 mg/kg ($P = 0.76$) and 0.02 mg/kg ($P = 0.63$), respectively, for each cigarette smoked. When cigarette smoking was categorized into two groups (i.e., non-smokers vs smokers), a similar pattern emerged where Fe_{COOK} was 0.86 mg/kg higher among smokers compared to non-smokers ($P < 0.0001$, Figure 20). Again, the same trend was observed after stratifying by ethnicity, where the magnitude of the association was greater for white females compared to black and Hispanic females (b1 = 0.81 mg/kg, $P < 0.0001$) vs 0.79 mg/kg ($P = 0.02$) and 0.49 mg/kg ($P = 0.13$), respectively, comparing Fe_{COOK} of smokers to that of non-smokers (Figure 21). When cigarette smoking was categorized into three groups (i.e., 0 cigarettes/d, 1-5 cigarettes/d or > 5 cigarettes/d), the same pattern emerged where Fe_{COOK} increased 0.53 mg/kg across each increasing category of

smoking ($P < 0.0001$, Figure 22). Moreover, the same trend was observed after stratifying by ethnicity, where the magnitude of the association was greater for white females compared to black and Hispanic females (b1 = 0.50 mg/kg, $P < 0.0001$) vs 0.54 mg/kg ($P = 0.008$) and 0.42 mg/kg ($P = 0.07$), respectively, with each increasing category of smoking (Figure 23).

Fe_{COOK} was inversely associated with physical activity (i.e., METS score), though the correlation was not statistically significant (r = -0.02, $P = 0.11$). The same pattern was observed among white and black females when stratifying by ethnicity (b1 = -0.006 mg/kg for each increasing unit of METS, $P = 0.71$ for white females; b1 = -0.005 mg/kg for each increasing unit of METS, $P = 0.86$ for black females). The pattern was opposite for Hispanic females (b1 = 0.07 mg/kg for each increasing unit of METS, $P = 0.05$).

Though dietary iron was positively associated with Fe_{COOK}, the correlation was weak and not statistically significant (r = 0.01, $P = 0.28$). In addition, increasing amounts of dietary iron did not have a substantial impact on Fe_{COOK} (b1 = 0.007 mg/kg, $P = 0.35$). Overall, the use of dietary supplements was inversely (but not significantly) associated with Fe_{COOK} (b1 = -0.03 mg/kg, $P = 0.87$), which was magnified when stratifying by ethnicity. Compared to white females, Fe_{COOK} was significantly inversely associated with iron supplementation among both Hispanic and black females (b1 = -1.03 mg/kg, $P = 0.04$ and b1 = -1.79 mg/kg, $P = 0.0004$). Taken together, iron, whether obtained from dietary or supplemental sources did not appear to be independently associated with iron stores in the current sample of females.

As above, the association between Fe_{COOK} and dietary/supplement intakes from foodstuffs considered as covariates in the current work are summarized below in terms of their effect on iron absorption (i.e., enhancers and inhibitors of iron absorption).

Promoters of iron absorption Overall, increased intakes of total saturated fats (in grams) obtained from the diet was positively associated with Fe_{COOK} (b1 = 0.004 mg/kg, P = 0.41). Given that the mean dietary intake of saturated fats was 22.56 grams per day, this corresponds to an increase in Fe_{COOK} of 0.09 mg/kg per day among those consuming the average amount of saturated fats in the sample. Increased intakes of palmitic and stearic saturated fatty acids were also positively associated with Fe_{COOK} (b1 = 0.006, P = 0.56 and b1 = 0.009 mg/kg, P = 0.62, respectively). In addition, the use of dietary supplements containing saturated fats was positively associated with Fe_{COOK} (b1 = 0.28 mg/kg, P = 0.63). Increasing intakes of dietary carbohydrates and sugars were inversely associated with Fe_{COOK} (b1 = -0.0006 mg/kg, P = 0.09 and b1 = -0.002, P = 0.03, respectively). In addition, use of supplements containing carbohydrates/sugars was inversely associated with Fe_{COOK} (b1 = -0.13, P = 0.71). Finally, increasing intakes of vitamin C was inversely associated with Fe_{COOK} (b1 = -0.0008 mg/kg, P = 0.25). Conversely, use of supplements containing vitamin C was positively associated with Fe_{COOK} (b1 = 0.16 mg/kg, P = 0.51). Taken together, promoters of iron absorption did not appear to be strongly associated with iron stores in this sample of females.

Inhibitors of iron absorption Intakes of dietary fiber were positively associated with Fe_{COOK} (b1 = 0.004 mg/kg, P = 0.51). Conversely, use of supplements containing fiber was inversely associated with Fe_{COOK} (b1 = -1.10 mg/kg, P = 0.01). Dietary and supplemental calcium were positively associated with Fe_{COOK} (b1 = 0.0002 mg/kg, P =

0.07 and b1 = 0.31, $P = 0.13$, respectively). Magnesium from dietary sources and supplements were both positively associated with Fe_{COOK} (b1 = 0.001 mg/kg, $P = 0.03$ and b1 = 0.17 mg/kg, $P = 0.47$, respectively). Intakes of vitamin D from dietary and supplemental sources were positively associated with Fe_{COOK} (b1 = 0.04 mg/kg, $P = 0.01$ and b1 = 0.25 mg/kg, $P = 0.28$, respectively). Dietary vitamin E intake was positively associated with Fe_{COOK} (b1 = 0.003 mg/kg, $P = 0.83$). Dietary and supplemental zinc intake was positively associated with Fe_{COOK} (b1 = 0.02, $P = 0.02$ and b1 = 0.20 mg/kg, $P = 0.42$). Taken together, the only inhibitor of iron absorption in the current sample of females which was associated with decreased iron stores was fiber obtained from supplements.

Total Body Iron and Reproductive Lifespan Mean Fe_{COOK} increased with self-reported age at menarche (b1 = 0.009 mg/kg, $P = 0.83$). When age at menarche was categorized as < 12 years of age, 12 years old or > 12 years of age, Fe_{COOK} increased with increasing age category: Fe_{COOK} $\ddot{x} = 6.93$ (sd = 5.63), $\ddot{x} = 6.94$ (sd = 3.90) and $\ddot{x} = 7.03$ (sd = 5.50), respectively, for each increasing age category ($P = 0.76$, Figure 24). A similar pattern emerged when stratified by ethnicity (Figure 25). While Fe_{COOK} increased across increasing categories for each ethnicity, no differences in Fe_{COOK} between ethnicities were detected ($P = 0.89$).

Fe_{COOK} increased with self-reported oral contraceptive use (Fe_{COOK} $\ddot{x} = 7.29$ (sd = 3.95) vs 6.32 (sd = 5.78), yes vs no) ($P < 0.0001$, Figure 26), decreased with increasing parity: 1 child, Fe_{COOK} $\ddot{x} = 7.59$, (sd = 4.45); 2-3 children, Fe_{COOK} $\ddot{x} = 7.04$ (sd = 4.81); > 3 children, Fe_{COOK} $\ddot{x} = 6.67$, (sd = 4.74), $P = 0.0009$, Figure 27), and increased among

post-menopausal females (Fe_{COOK} $\ddot{x}$ = 9.98 (sd = 3.34) vs Fe_{COOK} $\ddot{x}$ = 6.74 (sd = 4.87, P < 0.0001, Figure 28).

Metabolic dysfunction When defined using the criteria developed by the WHO, T2DM was present in 3.34% of the sample population (Table 3) and the mean age of females affected with T2DM was 38.52 (sd = 9.45). When stratified by ethnicity, T2DM was present in 2.64% of white, 3.79% of Hispanic and 3.95% of black females. When females were grouped into categories according to menopausal status, T2DM was present in 2.96% of premenopausal females and 10.58% of postmenopausal females (Table 3). When defined using the criteria developed by ATP III, metabolic syndrome was present in 15.71% of the sample (Table 3) and the mean age of those affected with metabolic syndrome was 35.78 (sd = 10.36). When stratified by ethnicity, metabolic syndrome was present in 13.83% of white, 14.82% of Hispanic and 14.08% of black females. When females were grouped into categories according to menopausal status, metabolic syndrome was present in 13.07% of premenopausal females and 34.01% of postmenopausal females (Table 3). When defined by the U.S.-FLI, the prevalence of NAFLD was 7.38% (Table 3) and the mean age of those affected with NAFLD was 31.49 (sd = 11.46). When stratified by ethnicity, the prevalence of NAFLD was 6.12% of white, 11.27% of Hispanic and 4.50% of black females. When females were grouped into categories according to menopausal status, 7.06% of premenopausal females had NAFLD and 11.58% of postmenopausal females had NAFLD (Table 3). When defined by HOMA-IR, the distribution of insulin resistance in the sample population was 64.06% - normal insulin resistance, 24.96% - moderate insulin resistance and 10.98% - severe insulin resistance (Table 3). The mean age of those with moderate to severe insulin

resistance was 27.74 (sd = 11.69). When stratified by ethnicity, the distribution of insulin resistance for white females was 73.23% - normal insulin resistance, 18.72% - moderate insulin resistance and 8.04% severe insulin resistance. For Hispanic females, the distribution of insulin resistance was 57.49% - normal insulin resistance, 29.35% - moderate insulin resistance and 13.16% - severe insulin resistance. The distribution of insulin resistance among black females was similar to that of Hispanic females, where 57.52% of females experienced normal insulin resistance, 29.51% experienced moderate insulin resistance and 12.96% experienced severe insulin resistance. When females were grouped into categories according to menopausal status (i.e., pre- vs. post-menopausal), 64.98% of premenopausal females experienced normal insulin resistance, 24.64% experienced moderate insulin resistance and 10.38% experienced severe insulin resistance (Table 3). Approximately sixty-three percent (i.e., 63.37%) of postmenopausal females experienced normal insulin resistance, 22.09% experienced moderate insulin resistance and 14.53% experienced severe insulin resistance (Table 3). Taken together, Hispanic and black females were disproportionately affected by T2DM, metabolic syndrome and insulin resistance. In addition, the prevalence of NAFLD was nearly two-fold higher in Hispanic females compared to their white counterparts and nearly three-fold higher compared to black females. Regardless of ethnicity, metabolic dysfunction was more prevalent among postmenopausal females compared to premenopausal females.

Total Body Iron and Metabolic Dysfunction The odds of T2DM increased with increasing Fe_{COOK} (OR = 1.03, 95%CI: 0.99-1.08, *P* = 0.15 for each unit increase in Fe_{COOK}. The effect was slightly attenuated after adjusting for covariates. When independently adjusting for 1) age, 2) ethnicity, 3) education, 4) BMI, 5) age at

menarche, 6) OC use, 7) parity, 8) menopausal status, 9) alcohol consumption, 10) smoking history, 11) physical activity and 12) C-reactive protein (CRP), the odds of T2DM were 1) 1.01 (95%CI: 0.98-1.05, $P = 0.43$), 2) 1.05 (95%CI: 1.00-1.09, $P = 0.05$), 3) 1.03 (95%CI: 0.99-1.08, $P = 0.16$), 4) 1.03 (95%CI: 0.98-1.07, $P = 0.24$), 5) 1.03 (95%CI: 0.98-1.08, $P = 0.25$), 6) 1.02 (95%CI: 0.97-1.07, $P = 0.40$), 7) 1.03 (95%CI: 0.98-1.08, $P = 0.21$), 8) 1.00 (95%CI: 0.95-1.05, $P = 0.94$), 9) 1.04 (95%CI: 0.99-1.09, $P = 0.12$), 10) 1.03 (95%CI: 0.99-1.08, $P = 0.17$), 11) 1.03 (95%CI: 0.99-1.08, $P = 0.16$) and 12) 1.02 (95%CI: 0.98-1.07, $P = 0.37$) (Table x), respectively. In a full model which adjusted for all covariates, the odds of T2DM increased with increasing Fe_{COOK}, though the effect size was slightly attenuated (OR = 1.02, 95%CI: 0.97-1.06, $P = 0.53$) (Table x). Given that ethnicity and menopausal status appeared to have the largest impacts as covariates, separate models were built to examine the interaction of each on the association between Fe_{COOK} and odds of T2DM. Compared to white females, the odds of T2DM among Hispanic and black females were 2.28 (95%CI: 1.65-3.14, $P < 0.0001$) and 2.42 (95%CI: 1.77-3.30, $P < 0.001$), respectively, for each unit increase in Fe_{COOK}. Taken together, it appears that non-white ethnicity is an effect modifier of Fe_{COOK} on the risk of T2DM, since ethnicity is more strongly associated with the outcome rather than the exposure. Compared to premenopausal females, the odds of T2DM among postmenopausal females were nearly 3-fold higher (OR = 2.96, 95%CI: 1.78-4.94, $P < 0.0001$).

When Fe_{COOK} was categorized according to tertiles, the odds of T2DM among those in the highest tertile of Fe_{COOK} compared to those in the lowest tertile were 1.30 (95%CI: 0.90-1.89, $P = 0.17$). A similar pattern was observed after stratifying by

ethnicity. Among white females, the odds of T2DM among those in the highest tertile of Fe_{COOK} compared to those in the lowest tertile were 1.11 (95%CI: 0.57-2.19, *P* = 0.75). The odds of T2DM were higher among Hispanic and black females in the highest tertile of Fe_{COOK} compared to those in the lowest tertile: OR = 1.52 (95%CI: 0.87-2.64, *P* = 0.14) and OR = 2.52 (95%CI: 1.39-4.57, *P* = 0.002. Finally, I examined the components of T2DM (i.e., fasting glucose concentration and HbA1C). Fe_{COOK} was linearly associated with fasting glucose concentration. For each unit increase in glucose concentration (md/dL), Fe_{COOK} increased 0.01 mg/kg (*P* = 0.01). Similarly, for each unit increase in $Fe_{COOK,}$ fasting glucose concentration increased 0.22 mg/dL (*P* = 0.01). Fe_{COOK} was inversely associated with HbA1C.For each unit (%) increase in HbA1C, Fe_{COOK} decreased 0.22 mg/kg (*P* = 0.02). Similarly, for each unit increase in $Fe_{COOK,}$ HbA1C decreased 0.005% (*P* = 0.02).

The odds of metabolic syndrome increased with increasing Fe_{COOK} (OR = 1.01, 95%CI: 0.99-1.04, *P* = 0.23 for each unit increase in $Fe_{COOK.}$ The effect was attenuated after adjusting for individual covariates and inverted in the full model. For example, when independently adjusting for 1) age, 2) ethnicity, 3) education, 4) BMI, 5) age at menarche, 6) OC use, 7) parity, 8) menopausal status, 9) alcohol consumption, 10) smoking history, 11) physical activity and 12) C-reactive protein (CRP), the odds of metabolic syndrome were 1) 1.00 (95%CI: 0.98-1.02, *P* = 0.92), 2) 1.02 (95%CI: 1.00-1.04, *P* = 0.13), 3) 1.01 (95%CI: 0.99-1.03, *P* = 0.36), 4) 1.01 (95%CI: 0.99-1.03, *P* = 0.53), 5) 1.01 (95%CI: 0.98-1.03, *P* = 0.65), 6) 1.00 (95%CI: 0.97-1.02, *P* = 0.86), 7) 1.01 (95%CI: 0.98-1.03, *P* = 0.54), 8) 0.99 (95%CI: 0.96-1.01, *P* = 0.37), 9) 1.01 (95%CI: 0.99-1.03, *P* = 0.36), 10) 1.01 (95%CI: 0.99-1.04, *P* = 0.32), 11) 1.01 (95%CI:

0.99-1.04, $P = 0.25$) and 12) 1.00 (95%CI: 0.98-1.02, $P = 0.73$) (Table x), respectively. In a full model which adjusted for all covariates, the effect size inverted to mirror the odds associated with Fe_{COOK} and metabolic syndrome when menopausal status was entered into the model (OR = 0.99, 95%CI: 0.96-1.01, $P = 0.28$) (Table x). Thus, a separate model was built to examine the interaction of menopausal status on the association between Fe_{COOK} and odds of metabolic syndrome. Compared to premenopausal females, the odds of metabolic syndrome among postmenopausal females was 2.7-fold higher (OR = 2.65, 95%CI: 2.02-3.47, $P < 0.0001$). Thus, menopausal status is an effect modifier of Fe_{COOK} on the risk of metabolic syndrome, since menopausal status is more strongly associated with the outcome rather than the exposure.

When Fe_{COOK} was categorized according to tertiles, the odds of metabolic syndrome among those in the highest tertile of Fe_{COOK} compared to those in the lowest tertile were 1.14 (95%CI: 0.94-1.39, $P = 0.18$). A similar pattern was observed after stratifying by ethnicity. Among white females, the odds of metabolic syndrome among those in the highest tertile of Fe_{COOK} compared to those in the lowest tertile were 1.08 (95%CI: 0.82-1.41, $P = 0.61$). The odds of metabolic syndrome were higher among Hispanic and black females in the highest tertile of Fe_{COOK} compared to those in the lowest tertile: OR = 1.28 (95%CI: 0.90-1.81, $P = 0.16$) and OR = 1.57 (95%CI: 1.15-2.16, $P = 0.005$). Finally, I examined the components of metabolic syndrome (i.e., fasting glucose concentration, triglycerides, HDL cholesterol, waist circumference and blood pressure). Among those with metabolic syndrome, Fe_{COOK} was linearly associated with fasting glucose concentration (b1 = 0.005 mg/kg, $P = 0.39$ for each mg/dL increase in glucose concentration), triglycerides (b1 = 0.001 mg/kg, $P = 0.0001$ for each mg/dL

increase in triglycerides), HDL cholesterol (b1 = 0.06 mg/kg, *P* = 0.52 for each mg/dL increase in HDL cholesterol) and waist circumference (b1 = 0.009 mg/kg, *P* = 0.44 for each cm increase in waist circumference). Among those with metabolic syndrome, Fe_{COOK} was inversely associated with systolic BP (b1 = -0.04 mg/kg, *P* = 0.002 for each unit increase in systolic BP (mmHg) and diastolic BP (b1 = 0.01 mg/kg, *P* = 0.41 for each unit increase in diastolic BP (mmHg).

Fe_{COOK} was linearly associated with U.S.-FLI. For each unit increase in U.S.-FLI, Fe_{COOK} increased 0.02 mg/kg (*P* = 0.001). Similarly, for each unit increase in Fe_{COOK}, U.S.-FLI increased 0.34 units (*P* = 0.003). The odds of NAFLD increased with increasing Fe_{COOK} (OR = 1.04, 95%CI: 1.00-1.08, *P* = 0.04 for each unit increase in Fe_{COOK}. The effect was attenuated when adjusting for covariates and abrogated in the full model. For instance, when independently adjusting for 1) age, 2) ethnicity, 3) education, 4) BMI, 5) age at menarche, 6) OC use, 7) parity, 8) menopausal status, 9) alcohol consumption, 10) smoking history, 11) physical activity and 12) C-reactive protein (CRP), the odds of NAFLD were 1) 1.02 (95%CI: 0.99-1.06, *P* = 0.11), 2) 1.04 (95%CI: 1.00-1.08, *P* = 0.02), 3) 1.04 (95%CI: 1.00-1.08, *P* = 0.03), 4) 1.01 (95%CI: 0.98-1.05, *P* = 0.48), 5) 1.04 (95%CI: 0.99-1.08, *P* = 0.09), 6) 1.03 (95%CI: 0.99-1.08, *P* = 0.12), 7) 1.03 (95%CI: 0.99-1.08, *P* = 0.09), 8) 1.03 (95%CI: 0.99-1.07, *P* = 0.22), 9) 1.02 (95%CI: 0.98-1.06, *P* = 0.31), 10) 1.02 (95%CI: 0.98-1.06, *P* = 0.35), 11) 1.02 (95%CI: 0.98-1.06, *P* = 0.31) and 12) 1.02 (95%CI: 0.99-1.06, *P* = 0.22) (Table x), respectively. In a full model which adjusted for all covariates, no increased odds of NAFLD as a function of Fe_{COOK} were observed (OR = 1.00, 95%CI: 0.96-1.04, *P* = 0.99) (Table x).

When Fe_{COOK} was categorized according to tertiles, the odds of NAFLD increased across increasing tertiles (P_{trend} = 0.04). Compared to those in the lowest tertile of Fe_{COOK} the odds of NAFLD were 1.05 (95%CI: 0.77-1.44, P = 0.74) for those in the middle tertile of Fe_{COOK}. Compared to those in the lowest tertile of Fe_{COOK}, the odds of NAFLD were 1.35 (95%CI: 1.00-1.81, P = 0.05). A similar pattern was observed after stratifying by ethnicity. Among white females, the odds of NAFLD among those in the highest tertile of Fe_{COOK} compared to those in the lowest tertile were 1.52 (95%CI: 1.00-2.31, P = 0.05). The odds of NAFLD were higher among Hispanic females in the highest tertile of Fe_{COOK} compared to those in the lowest tertile: OR = 1.10 (95%CI: 0.69-1.75, P = 0.69). Compared to their white and Hispanic counterparts, the odds of NAFLD were higher among black females in the highest tertile of Fe_{COOK} compared to those in the lowest tertile: OR = 1.62 (95%CI: 0.88-3.01, P = 0.12). Fe_{COOK} was also linearly associated with liver fibrosis (i.e., NFS). For each unit increase in NFS, Fe_{COOK} increased 0.42 mg/kg (P < 0.0001). Similarly, for each unit increase in Fe_{COOK}, NFS increased 0.05 units (P < 0.0001).

Fe_{COOK} was linearly associated with insulin resistance. For each unit increase in HOMA-IR, Fe_{COOK} increased 0.02 mg/kg (P = 0.41). Similarly, for each unit increase in Fe_{COOK}, HOMA-IR increased 0.01 units (P = 0.43). The odds of insulin resistance increased with increasing Fe_{COOK} (OR = 1.04, 95%CI: 1.00-1.08, P = 0.05 for each unit increase in Fe_{COOK}. The effect was not attenuated after adjusting for covariates. When independently adjusting for 1) age, 2) ethnicity, 3) education, 4) BMI, 5) age at menarche, 6) OC use, 7) parity, 8) menopausal status, 9) alcohol consumption, 10) smoking history, 11) physical activity and 12) C-reactive protein (CRP), the odds of

insulin resistance were 1) 1.04 (95%CI: 1.00-1.08, *P* = 0.04), 2) 1.05 (95%CI: 1.01-1.09, *P* = 0.01), 3) 1.04 (95%CI: 1.00-1.08, *P* = 0.02), 4) 1.03 (95%CI: 0.99-1.06, *P* = 0.20), 5) 1.04 (95%CI: 1.00-1.08, *P* = 0.04), 6) 1.04 (95%CI: 1.00-1.08, *P* = 0.03), 7) 1.04 (95%CI: 1.00-1.08, *P* = 0.04), 8) 1.04 (95%CI: 0.99-1.07, *P* = 0.07), 9) 1.03 (95%CI: 0.99-1.08, *P* = 0.12), 10) 1.03 (95%CI: 0.99-1.07, *P* = 0.17), 11) 1.03 (95%CI: 0.99-1.07, *P* = 0.22) and 12) 1.02 (95%CI: 0.98-1.06, *P* = 0.27) (Table x), respectively. In a full model which adjusted for all covariates, the effect size persisted. However, the effect was no longer statistically significant (OR = 1.04, 95%CI: 1.00-1.08, *P* = 0.07) (Table x).

When Fe_{COOK} was categorized according to tertiles, the odds of insulin resistance increased across increasing tertiles (P_{trend} = 0.10). Compared to those in the lowest tertile of Fe_{COOK} the odds of insulin resistance were 1.23 (95%CI: 0.86-1.78, *P* = 0.26) for those in the middle tertile of Fe_{COOK}. Compared to those in the lowest tertile of Fe_{COOK}, the odds of insulin resistance were 1.46 (95%CI: 1.03-2.06, *P* = 0.03) among those in the highest tertile of Fe_{COOK}. A similar pattern was observed after stratifying by ethnicity, especially among white females. Among white females, the odds of insulin resistance among those in the highest tertile of Fe_{COOK} compared to those in the lowest tertile were 2.59 (95%CI: 1.45-4.65, *P* = 0.005). The odds of insulin resistance were also higher among Hispanic and black females in the highest tertile of Fe_{COOK} compared to those in the lowest tertile: OR = 1.04 (95%CI: 0.60-1.78, *P* = 0.58) and OR = 1.25 (95%CI: 0.80-1.96, *P* = 0.47), respectively.

CHAPTER 7: DISCUSSION, LIMITATIONS AND CONCLUSIONS

The aim of the current dissertation was to evaluate iron stores in a nationally-representative, community-dwelling, non-institutionalized group of females participating in continuous NHANES from 2003 to 2010 using an index of iron repletion developed by JD Cook et al. in 1993 (i.e., Fe_{COOK}) (50). I first sought to describe the relationships between Fe_{COOK} and age, ethnicity, education, BMI, physical activity, alcohol intake and smoking status in the female cohort. I hypothesized that Fe_{COOK} would be positively associated with Hispanic and Non-Hispanic Caucasian ethnicity, education, BMI, alcohol consumption, smoking and negatively associated with Non-Hispanic African American ethnicity and physical activity. The results confirm that mean Fe_{COOK} in all females in the current sample was higher than the reference mean for the index (i.e., $\ddot{x} = 5.5 \pm 3.4$ mg/kg) (50), indicating that these females were iron-replete. This finding aligns with several recent large prospective studies which indicate that most of the U.S. population is iron-replete. In the current sample, Hispanic and white females had higher Fe_{COOK} than black females, with white females having higher body iron than other ethnicities. Moreover, Fe_{COOK} increased with increasing age, education, BMI, alcohol consumption and smoking history among females in the sample and decreased with increasing physical activity among white and black females. Therefore, it appears that Fe_{COOK} as an index of iron repletion demonstrates clinical utility in assessing the association between iron stores

and demographic variables in the current sample of U.S. females. An unexpected finding was that Fe_{COOK} increased with increasing physical activity among Hispanic females.

Next, my aim was to examine the distribution of Fe_{COOK} in the total sample after stratifying participants on several variables related to the female reproductive lifespan. Specifically, I hypothesized that Fe_{COOK} would be positively associated with use of oral contraceptives and decreasing age at menopause and negatively associated with age at menarche and parity. The results confirm that Fe_{COOK} increased with oral contraceptive use, decreased with decreasing age at menarche and decreased with increasing parity, highlighting again the practical use (i.e., clinical utility) of the index in assessing the association between iron stores and reproductive parameters among U.S. females.

It should be noted, however, that the current results do not support the hypothesis that Fe_{COOK} increases with an earlier age at menopause. This could be due to several factors, including the age distribution among females in the sample. As mentioned, approximately 43% (i.e., 42.9%) of the sample were between the ages of 12 and 21 compared to 17% who were aged 42 years or older. Moreover, while the mean age of females in the current sample was 27.21 years, the mode was 14 years of age. In addition, NHANES did not collect data on the actual self-reported age at menopause among females. Rather, the survey inquired as to what age each participant experienced her last menstrual cycle. Given that this question was not age-restricted, nor was it associated with any obvious any skip-pattern, one must infer that this question applied to all females in the sample. In other words, the age at which a given participant experienced her 'last' menstrual cycle could have very well been the same age at which she was interviewed. Thus, defining age at menopause based upon this NHANES variable would have

obviously lead to biased results. Thus, menopausal status was ascertained in the current sample by utilizing two additional survey questions (i.e., one which inquired as to whether the participant had at least one period in the past twelve months and another which inquired as to the reason why the participant had not had a period in the past twelve months). When the age distribution of females who answered 'no' to the former question and 'menopause/hysterectomy' to the latter question was examined, Fe_{COOK} was positively associated with age. This is not surprising, given that this particular finding was derived from the self-reported age during the time of interview among females who were subsequently scored as postmenopausal.

I then sought to estimate the prevalence of NAFLD among females in the current sample using a validated algorithm developed by Ruhl et al. in 2015, the U.S. fatty liver index (U.S.-FLI). Overall, the prevalence of NAFLD among females in the sample was low (i.e., 7.38% in all females, 6.12% in white females, 11.27% in Hispanic females and 4.50% in black females). As mentioned, NAFLD is rarely observed in individuals younger than 20 years (~1%), but increases with age (~18% in those aged 20-40 years to 39% among adults aged 60 and older) (442). Given the age distribution among females in the current sample, these data are in agreement with the epidemiological literature. Moreover, the prevalence of NAFLD observed across ethnicities in the current sample is in accord with previous reports. For example, investigators from the Dallas Heart Study have reported that the prevalence of NAFLD was higher in Hispanic Americans compared to other ethnicities. Moreover, the same study demonstrated that African Americans had the lowest prevalence of NAFLD (443). Taken together, these results are in accord with the literature.

Next, my aim was to confirm and extend associations between iron stores, in particular Fe_{COOK}, and metabolic dysfunction reported in the epidemiological literature. I hypothesized that Fe_{COOK} would be positively associated with T2DM, metabolic syndrome, NAFLD and insulin resistance after controlling for age, BMI, alcohol consumption, smoking status, physical activity and micronutrient status. When metabolic dysfunction was modeled as a function of continuously-scaled Fe_{COOK}, the current work confirms a positive association between Fe_{COOK} and T2DM and insulin resistance but not for metabolic syndrome or NAFLD after adjustment for covariates. All effect sizes were small and not statistically significant. However, while the magnitude of associations between continuously-scaled Fe_{COOK} and metabolic dysfunction was small, the direction of the observed associations supports previous work (395,396). It is worth noting that T2DM, metabolic syndrome and NAFLD are all diseases which develop over several decades. As mentioned, the prevalence of T2DM, metabolic syndrome and NAFLD among individuals < 20 years of age is < 1%, ~7% and < 1%, respectively. Therefore, it should be expected that the magnitude of association between continuously-scaled Fe_{COOK} and metabolic dysfunction in the current sample be small.

The current results also support previous work demonstrating independent associations between ethnicity and menopausal status and risk of metabolic dysfunction. In other words, when continuously-scaled Fe_{COOK} was used to model metabolic dysfunction, it appears that ethnicity and menopausal status modified the effect of Fe_{COOK} on the risk of T2DM and metabolic syndrome among females in this sample, indicating that these two variables were significantly and independently associated with each outcome.

Finally, and most importantly, the results between continuously-scaled Fe_{COOK} and metabolic dysfunction are not surprising based upon trends in reporting commonly found in the literature, where nearly all associations between iron stores (i.e., serum ferritin, TSAT) and metabolic dysfunction have modeled iron stores categorically (e.g., tertiles, quartiles, quintiles). Thus, the current results align more closely with what has been published when Fe_{COOK} was also modeled categorically. For example, females in the highest tertile of Fe_{COOK} compared to those in the lowest tertile of Fe_{COOK} had significantly increased odds of T2DM, metabolic syndrome, NAFLD and insulin resistance (OR = 1.30, 95%CI:0.90-1.89, P_{trend} = 0.17 for T2DM, OR = 1.14, 95%CI: 0.94-1.39, P_{trend} = 0.18 for metabolic syndrome, OR = 1.35, 95%CI: 1.00-1.81, P_{trend} = 0.05 for NAFLD and OR = 1.46, 95%CI: 1.03-2.06, P_{trend} = 0.03). Differential risk patterns for each outcome emerged across ethnicities when categorizing Fe_{COOK} in this manner. Black females in the highest tertile of Fe_{COOK} compared to those in the lowest tertile of Fe_{COOK} had significantly increased odds of T2DM compared to their Hispanic and white counterparts. This does not align with prevalence estimates reported in the literature, which indicate that Hispanic females have the highest risk of T2DM and metabolic syndrome, followed by black females and white females. One might speculate that the discordance between the current results and what has been reported in the literature is due to Hispanic females in the current sample consuming 3.25 g/d more dietary fiber (i.e., an inhibitor of iron absorption) than black females. Moreover, given that all females were iron-replete, the decreased risk of T2DM, metabolic syndrome and NAFLD among white females could have been moderated by increased physical activity levels in this group. In addition, the increased risk of T2DM, metabolic syndrome and

NAFLD among black females could have been moderated by increased intake of dietary sugars and saturated fat in this group, where the former has been shown to increase iron absorption and the latter increases siderophores.

A strength of the current work is that I used a large, ethnically-diverse and nationally-representative sample of U.S. females. Therefore, the current findings should be generalizable at the population level. Moreover, the standardized methodology employed in the quantification of Fe_{COOK} and outcomes of the study permits replication of each finding, as NHANES data is available for public use. Another strength of the study is that the clinical validity of the index developed by Cook et al. was highlighted compared to TSAT when examining the associations between each index and metabolic dysfunction (Appendix 2). While TSAT is widely used in the clinical setting to assess iron stores, it was previously highlighted that it is unreliable due to diurnal variation in serum iron concentrations (i.e., the numerator of TSAT). Moreover, only a small percentage of total body iron is accounted for by TSAT (~1%, compared to 20% with ferritin) (109,110). As also mentioned, inflammation and the use of oral contraceptives decreases total iron binding capacity (i.e., the denominator of TSAT) which would ultimately lead to lower TSAT among females who use them. To this end, most of the females in the current sample reported using oral contraceptives (53.11%) and white females had the highest self-reported usage of oral contraceptives compared to Hispanic and black females (53.50% vs. 25.14% and 21.36%, respectively). It is important to mention these proportions for several reasons. First, if one assumes that the use of oral contraceptives influences TSAT, then it appears that the odds associated with each outcome when TSAT was categorized according to tertiles track with these proportions

(Table 13). Second, if one also considers the mean age of females in the sample with T2DM (38.52, sd = 9.45), metabolic syndrome (35.78, sd = 10.36), NAFLD (31.49, sd = 11.46) and insulin resistance (27.74, sd = 11.69) with the mean age of oral contraceptive users (i.e., 33.07 (sd = 9.94)), it is tempting to hypothesize that the reduced odds of each outcome with increasing tertile of TSAT is also related to the use of oral contraceptives. An alternative explanation is that increasing iron deficiency accounts for the increased risk of metabolic dysfunction among those in the lowest tertile of TSAT. Otherwise, females in the highest tertile of TSAT would not have had a decreased risk of metabolic dysfunction compared to those in the lowest tertile of TSAT. Given the average TSAT among females in the current sample was 21.84% and that a TSAT < 20% is typically the threshold used in the clinical setting to distinguish iron deficiency, it doesn't appear that females in the current sample were iron deficient. Therefore, it doesn't appear that increasing iron deficiency was associated with an increased risk of metabolic dysfunction, which is what the observed effect sizes indicate. A simpler explanation is that TSAT among females in the current sample was adversely affected by the aforementioned limitations of the index, particularly oral contraceptive use. Theoretically, oral contraceptive use should increase iron stores (i.e., serum ferritin concentrations) due to the cessation of menstruation. The results obtained in the current work using the index developed by Cook et al supports this theory, where Fe_{COOK} increased with increasing OC use. Given that Fe_{COOK} is unaffected by diurnal variation, accounts for a larger fraction of body iron stores and is unaffected by inflammation or use of oral contraceptives, it is reasonable to conclude that the clinical utility and clinical validity of the index was highlighted in the current study.

There are several limitations in the current work which must be noted. First, given the cross-sectional study design of NHANES, one cannot infer causal relationships based upon the current results. Second, a key variable used in the derivation of Fe_{COOK} (i.e., serum transferrin receptor) was not collected among male participants or females aged greater than 49 years of age. This is discouraging, as much of the current research supporting an association between iron stores and metabolic dysfunction has been obtained from studies using males and/or older (i.e., postmenopausal) females. As most of the females in the sample were young (mode = 14), it is possible that no significant associations between iron stores and metabolic dysfunction were observed due to regular menstruation. Moreover, the development of each outcome can take decades to manifest. In line with the latter association, I was able to detect a positive association between increasing tertiles of Fe_{COOK} and all outcomes among postmenopausal females when Fe_{COOK} was modeled in accordance with common reporting patterns in the current literature (Table 14), though no comparison was statistically significant due to such a small number of postmenopausal females in the current study.

Another limitation of the current work is that many females did not have complete data for biochemical parameters required in the derivation of each outcome. Thus, the sample size for each outcome differed from the number of females in the full sample and it is therefore possible that 1) the low prevalence of metabolic dysfunction might have been an artefact of missing data and 2) the original power calculations were overestimates. The latter is a limitation, as post hoc power analyses were not conducted in the current work. Moreover, the reduced and variable sample sizes for each outcome may have been affected by the PROC SURVEY procedures employed to obtain results

that could generalize to the population. It would have been interesting to model the data using standard SAS procedures. While one could not generalize to the population, different patterns may have been observed among females in the current sample.

In conclusion, the current results highlight the clinical utility of Fe_{COOK} in assessing the iron status in females of reproductive age. I did not observe a statistically significant association between metabolic dysfunction and continuously-scaled Fe_{COOK}. However, when Fe_{COOK} was modeled in accordance with common reporting patterns (i.e., where iron stores are modeled categorically), significant, positive associations between increasing Fe_{COOK} and metabolic dysfunction were observed among all females, and this pattern persisted after stratification by ethnicity and menopausal status. Further research is warranted to examine the clinical utility of Fe_{COOK} among other cohorts. Moreover, additional research using Fe_{COOK} is recommended in order to replicate these findings among a larger sample comprised of both males and older females.

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Figure 1: Flowchart of sample N's.

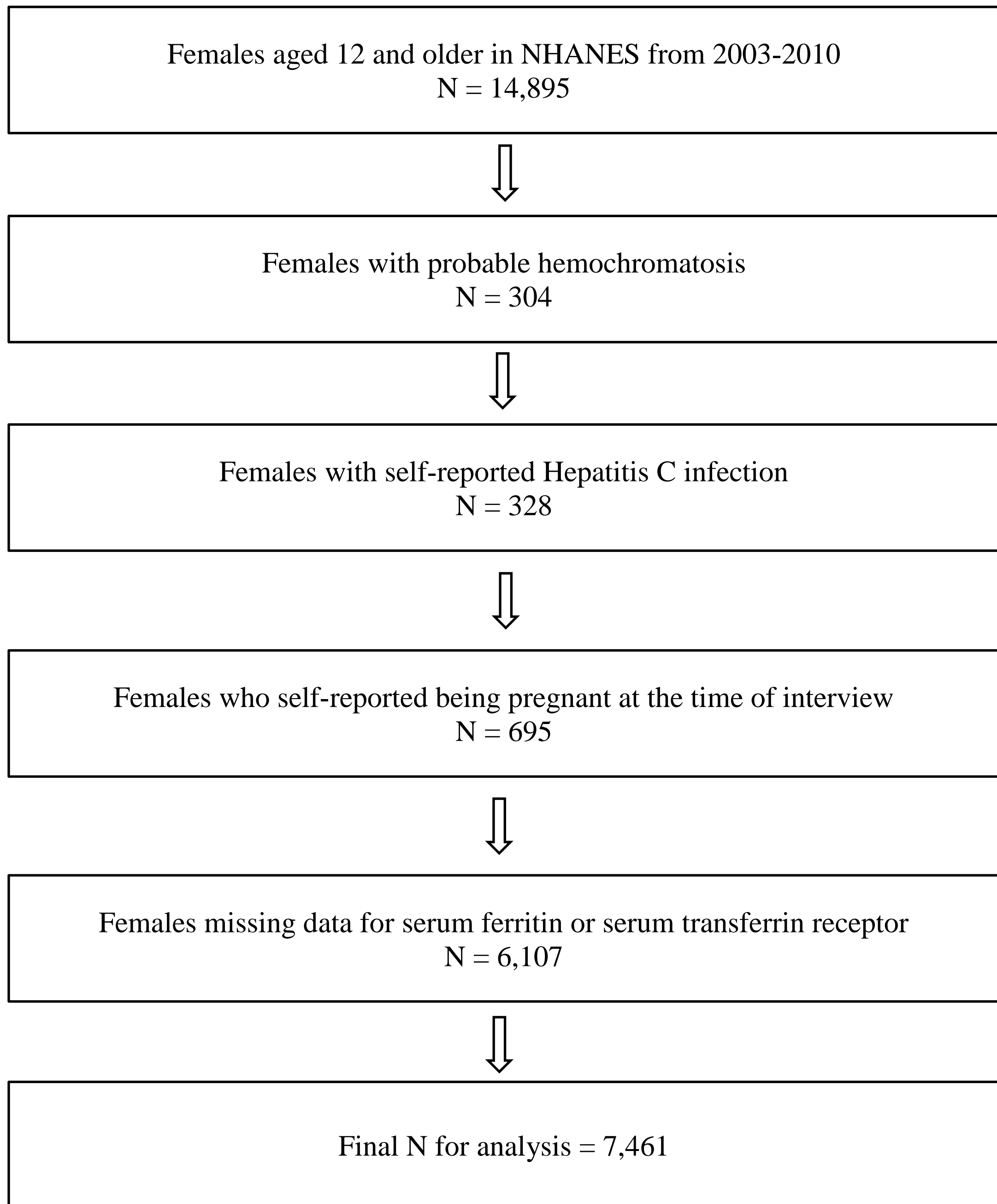


Table 1: Demographics of female participants: continuous NHANES 2003-2010 (N = 7,461)		
Variable	Value	N (%)
Age (yr)	12-21	3,200 (42.89)
	22-31	1,490 (19.97)
	32-41	1,504 (20.16)
	42-49	1,267 (16.98)
Ethnicity	White	3,182 (42.65)
	Black	1,846 (24.76)
	Hispanic	2,432 (32.30)
Education	< High school diploma	3,952 (52.97)
	High school diploma / GED	986 (13.22)
	Some college	1,542 (20.43)
	College degree / post graduate degree	995 (13.34)
Waist circumference	Mean (sd)	89.33 (16.85)
Body mass index	< 18.5	403 (5.46)
	18.5 – 24.9	3,039 (41.21)
	25.0 – 29.9	1,761 (23.88)
	> 29.9	2,172 (29.45)
Alcohol Consumption	0 drinks/d	4,635 (62.14)
	1-3 drinks/d	2,259 (30.29)
	> 3 drinks/d	565 (7.57)
Smoking Status	0 cigarettes/d	6,138 (82.29)
	1-5 cigarettes/d	506 (6.78)
	> 5 cigarettes/d	815 (10.93)
* N's do not sum to 7,461 due to missing values for given variable		

Table 2: Variables associated with reproductive lifespan of female participants: continuous NHANES 2003-2010 (N = 7,461)		
Variable	Value	N (%)
Age at menarche	Mean (sd)	12.36 (1.61)
Age at menarche category*	< 12 y	1,813 (27.75)
	12 y	1,851 (28.33)
	> 12 y	2,870 (43.92)
Oral Contraceptive Use*	No	3,063 (46.89)
	Yes	3,469 (53.11)
Parity*	0	3,235 (49.51)
	1	655 (10.02)
	2-3	1,628 (24.92)
	> 3	1,016 (15.55)
Age at menopause	Mean (sd)	43.94 (4.80)
Age at menopause category*	< 40 y	61 (16.99)
	40-45 y	127 (35.28)
	> 45 y	171 (47.63)
* N's do not sum to 7,461 due to missing values for given variable		

Table 3: Biochemical parameters of female participants: continuous NHANES 2003-2010			
Variable	All females, mean (sd)	Premenopausal females, mean (sd)	Postmenopausal females, mean (sd)
Hemoglobin (g/dL)	13.44 (2.96)	13.42 (2.64)	13.88 (1.10)
Hematrocrit (%)	39.39 (9.09)	39.30 (8.02)	40.64 (3.75)
Serum Iron (µg/dL)	77.42 (48.74)	77.72 (47.89)	79.61 (39.34)
TIBC (µg/dL)	365.02 (102.28)	366.06 (104.61)	340.57 (43.57)
TSAT (%)	21.84 (13.68)	21.80 (12.86)	23.33 (11.57)
sTfR (mg/L)	3.59 (2.47)	3.60 (2.43)	3.27 (1.56)
Ferritin (µg/L)	47.94 (47.47)	44.73 (45.93)	86.28 (50.75)
Serum Insulin (uIU/mL)	11.67 (13.61)	11.46 (13.44)	11.12 (5.66)
Serum Glucose (mmol/L)	5.27 (1.41)	5.25 (1.40)	5.52 (0.96)
GGT (U/L)	18.92 (29.63)	18.01 (26.45)	28.88 (40.07)
Platelets (1000 cells/uL)	284.75 (118.27)	283.07 (117.33)	287.15 (86.42)
Albumin (g/dL)	4.21 (0.62)	4.21 (0.62)	4.20 (0.43)
Triglycerides (mg/dL)	106.87 (138.58)	104.82 (146.83)	139.63 (55.40)
HDL cholesterol (mg/dL)	57.62 (36.17)	57.53 (34.33)	57.82 (17.69)
HbA_{1c} (%)	5.28 (0.78)	5.25 (0.80)	5.53 (0.84)
C-reactive protein (mg/dL)	0.39 (0.96)	0.38 (0.98)	0.51 (0.54)

Table 4: Predictor and Outcome Variables				
Variable		All females, mean (sd) or tertile/category cutoff %	Premenopausal females, mean (sd) or tertile/category cutoff %	Postmenopausal females, mean (sd) or tertile/category cutoff %
Fe_{COOK} mean (SD)		6.98 (4.82)	6.74 (4.87)	9.98 (3.34)
Fe_{COOK}	White	7.27 (4.28)	7.02 (4.21)	10.03 (2.36)
	Hispanic	6.51 (6.81)	6.31 (6.25)	10.33 (1.68)
	Black	5.95 (4.24)	5.70 (5.33)	9.46 (2.49)
HOMA-IR	< 3	64.06%	65.14%	62.05%
	3 - 5	24.96%	24.25%	22.56%
	> 5	10.98%	10.61%	15.38%
U.S.-FLI	< 30	92.62%	92.94%	88.42%
	≥ 30	7.38%	7.06%	11.58%
NFS	≤ 0.676	99.63%	99.70%	97.87%
	> 0.676	0.37%	0.30%	2.13%
Metabolic Syndrome	Absent	84.29%	85.31%	69.52%
	Present	15.71%	14.69%	30.48%
T2DM	Absent	96.66%	97.08%	91.78%
	Present	3.34%	2.92%	8.22%

Table 5: Common iron parameters with reference ranges		
Iron Parameter	Lab Test / Calculation	Typical Range of Values / Reference Range
Hemoglobin (Hb)	Colorimeter	Males: 14 - 18 g/dL Females: 12 - 16 g /dL
Hematocrit (Hct)	Automated cell counter: (Red Cell Number/MCV) or Indirect: (packed red cells/1000 ml of whole blood)	Range: 0 - 100% Males: 40 - 54% Females: 36 - 48%
Hepcidin	Immunoassay	Males: 0.6 – 23.3 nM Females: 0.5 – 23.2 nM
Mean corpuscular volume (MCV)	(Volume of packed red cells/1000 mL of whole blood) / (red cell count in millions/mL)	80 – 100 fL
Mean corpuscular hemoglobin (MCH)	(Hb (g) / 1000 mL whole blood) / (red cell count in millions/mL)	26 – 34 pg/cell
Mean corpuscular hemoglobin concentration (MCHC)	(Hb (g) / 1000 mL whole blood) / (Volume of packed red cells/1000 mL of whole blood)	31 -37 g/dL
RDW	Whole Blood	11.5 – 14.5%
Glycosylated Hb	Whole Blood	4 – 6%
Serum Iron (μg/dL)	Whole Blood	40 – 180 mcg/dL

Table 5 (continued): Common iron parameters with reference ranges		
Transferrin	Whole Blood	170 – 370 mg/dL
TIBC (µg/dL)	Whole Blood	250 – 450 mcg/dL
TSAT (%)	Serum Iron*(100)/TIBC	Range: 0-100% 25 – 35%
sTfR (mg/L)	Immunoassay	5.6 ± 0.3 mcg/L
Ferritin (µg/L)	Immunoassay	Males: 20 – 300 ng/mL Females: 15 – 200 ng/mL
Fe_{COOK} (mg/kg)	$(-[\log10(R*1000/F) - 2.8229])/0.1207$	Range: -20 – 20 Mean = 5.5 ± 3.4 mg/kg
sTfR:Ferritin Index	sTfR/log(F)	Range: 1 – 10 Mean = 4.1 ± 1.7
Log(sTfR)/Ferritin ratio	log(sTfR)/F	Range: 1 – 10 Mean = 2.3 ± 0.3

Table 6a: Iron-loading conditions (primary iron overload)

Primary iron overload						
Iron loading disorder	Genetic Mutation	Locus	Mode of Inheritance	Mechanism	Affected Iron Parameters	Disease association/clinical features
HFE-related hemochromatosis	HFE	6p21	Autosomal recessive	Low hepcidin	High serum iron, TSAT, ferritin Low TIBC, StfR	Parenchymal iron overload, liver disease, hepatocellular carcinoma, endocrinopathy, diabetes, arthropathy
TFR2-related hemochromatosis	TFR2	7q22	Autosomal recessive	Low hepcidin	High TSAT, ferritin	Parenchymal iron overload, liver disease
FPN-related hemochromatosis	SLC40A1 (FPN1)	2q32	Autosomal dominant	Reduced iron export from macrophages, hepcidin resistance	High ferritin Low to normal TSAT	Parenchymal and reticuloendothelial iron overload
Juvenile hemochromatosis 2a	HJV	1q21	Autosomal recessive	Reduced HAMP activation leading to low hepcidin	High TSAT, ferritin	Severe parenchymal iron overload, cardiovascular disease, liver disease, endocrinopathy, diabetes, arthropathy
Juvenile hemochromatosis 2b	HAMP	19q13	Autosomal recessive	Low to absent hepcidin	High TSAT, ferritin	Severe parenchymal iron overload, cardiovascular disease, liver disease, endocrine failure diabetes, arthropathy

Adapted from 178-181

Table 6b: Iron-loading conditions (ineffective erythropoiesis)

Secondary iron overload: ineffective erythropoiesis						
Iron loading disorder	Genetic Mutation	Locus	Mode of Inheritance	Mechanism	Affected Iron Parameters	Disease association/clinical features
Beta thalassemia	HBB			Low to normal hepcidin	High serum iron, TSAT, ferritin, StfR Low TIBC	Parenchymal and reticuloendothelial iron overload
Sickle cell anemia	HBB			Low to normal hepcidin	High serum iron, TSAT, ferritin Low TIBC	Parenchymal and reticuloendothelial iron overload
X-linked sideroblastic anemia	ALAS2			Low hepcidin	High serum iron, TSAT, ferritin Low TIBC	Parenchymal and reticuloendothelial iron overload

Adapted from 178-181

Table 6c: Iron-loading conditions (chronic hemolytic anemias)

Secondary iron overload: Chronic hemolytic anemias						
Iron loading disorder	Genetic Mutation	Locus	Mode of Inheritance	Mechanism	Affected Iron Parameters	Disease association/clinical features
Pyruvate-kinase deficiency	PKLR			Low hepcidin	High serum iron, TSAT, ferritin Low TIBC	Parenchymal iron overload, anemia
Hereditary spherocytosis	Heterogeneous			Low hepcidin	High serum iron, TSAT, ferritin Low TIBC	Parenchymal anemia, jaundice, splenomegaly

Adapted from 178-181

Table 6d: Iron-loading conditions (hypoplastic anemias)

Secondary iron overload: hypoplastic anemias						
Iron loading disorder	Genetic Mutation	Locus	Mode of Inheritance	Mechanism	Affected Iron Parameters	Disease association/clinical features
Fanconi anemia	FANC group			Low hepcidin		Pancytopenia, myelodysplastic syndromes
Diamond-Blackfan anemia	Heterogeneous			Low hepcidin		Red cell aplasia, predisposition to leukemia
Chronic renal failure				High hepcidin		Iron loading, anemia due to ineffective erythropoiesis

Adapted from 178-181

Table 6e: Iron-loading conditions (other iron loading conditions)

Other iron-loading disorders						
Iron loading disorder	Genetic Mutation	Locus	Mode of Inheritance	Mechanism	Affected Iron Parameters	Disease association/clinical features
Friedreich ataxia	FXN			Unknown	High serum iron, TSAT, ferritin Low TIBC	Mitochondrial iron overload, Neurological disease, cardiovascular disease
Hereditary atransferrinemia	TF	3q21	Autosomal recessive	Low hepcidin	High TSAT, ferritin Low serum iron	Liver disease, cardiovascular disease
Hereditary aceruloplasminemia	CP	3q23-q24	Autosomal recessive	Low hepcidin	High ferritin Low serum iron, TSAT	Neurological disease, retinal degeneration, diabetes

Adapted from 178-181

Table 7: Comparison of non-invasive measure for hepatic steatosis

NALFD estimate	Individual Components	Sensitivity	Specificity	AUC
Biopsy	Liver sample	ref	ref	ref
Abdominal Ultrasound (AUS)	Ultrasound of Liver	49.1%	75%	N/A
Hepatorenal Ultrasound Index (HRI)	Ratio between median brightness level of the liver and cortex of the right kidney	Cutoff ≥ 1.5 (representing 5% fatty liver), 100% (vs biopsy) (Webb)	Cutoff ≥ 1.5 (representing 5% fatty liver), 91% (vs biopsy) (Webb)	0.99
AST-to-platelet ratio index (APRI)	Aspartate aminotransferase / platelet count	89% (Adler)	75% (Adler)	0.82 (Adler)
AST-to-ALT ratio	Aspartate aminotransferase, alanine aminotransferase ratio	53%	100%	N/A
Original European Liver Fibrosis panel (OELF) / Enhanced Liver Panel (ELF)	Age, hyaluronic acid, n-terminal propeptide of type III collagen, tissue inhibitors of matrix metalloproteinase-1	Cutoff > 0.375, 89% (Rosenberg)	Cutoff > 0.375, 96% (Rosenberg)	0.87 (Rosenberg) 0.80 (Nobili)
		90% (Nobili)	41% (Nobili)	

Table 7 (con't): Comparison of non-invasive measure for hepatic steatosis

NALFD estimate	Individual Components	Sensitivity	Specificity	AUC
Fatty Liver Index (FLI)	triglycerides, body mass index, gamma glutamyl transferase	Cutoff > 20, 62% vs LAP (yang)	Cutoff > 20, 86% vs LAP (yang);	Females, 0.83 (0.82-0.83) (yang)
		Cutoff ≥ 60, 87% (Bedogni)	Cutoff ≥ 60, 86% (Bedogni)	0.84 (Bedogni) 83 (Huang) 81% (Koehler)
		Cutoff > 30, 80% Huang	Cutoff > 30, 72% (Huang)	
		Cutoff = 60, 62% (Koehler)	Cutoff = 60, 81% (Koehler)	
FIB-4 index	Platelet count, aspartate aminotransferase, alanine aminotransferase, age	Cutoff = 1.3, 85% (McPherson) 70% (Shah)	Cutoff = 1.3, 65% (McPherson) 74% (Shah)	0.86 (McPherson) 0.80 (Shah)
FibroIndex	Platelet count, aspartate aminotransferase, gamma glutamyl transferase	78%	74%	N/A
FibroMeter	Platelet count, gamma-2 macroglobulin, aspartate aminotransferase, age, prothrombin index, hyaluronic acid, blood urea nitrogen	Cutoff = 0.61, 79% (Cales)	Cutoff = 0.61, 96% (Cales)	0.94 (Cales)
Fibrospect II	Hyaluronic acid, tissue inhibitors of matrix metalloproteinase-1, alpha-2 macroglobulin	83.5%	66.7%	N/A
FibroTest	gamma-2 macroglobulin, apolipoprotein A1, gamma glutamyl transferase, total bilirubin	Fibrosis cutoff score ≥ 2, 77% (Ratziu)	Fibrosis cutoff score ≥ 2, 98% (Ratziu)	0.86 (Ratziu)
Forns	Age, platelet count, gamma glutamyl transferase, cholesterol level	94% (Adler)	51% (Adler)	0.86 (Adler)

Table 7 (con't): Comparison of non-invasive measure for hepatic steatosis

NALFD estimate	Individual Components	Sensitivity	Specificity	AUC
Hepascore	Age, gender, bilirubin, gamma glutamyl transferase, hyaluronic acid, gamma-2 macroglobulin	63%	89%	N/A
Lipid Accumulation Products (LAP)	Waist circumference, Triglycerides	Cutoff = 31.6, 66% (Chiang)	Cutoff = 31.6, 93% (Chiang)	0.87 (Chiang) Females, 0.794 (0.786-0.802) (yang)
PGA index	Prothrombin index, gamma glutamyl transferase, apolipoprotein A1	91%	81%	N/A
SHASTA index	Hyaluronic acid, aspartate aminotransferase, albumin	100%	52%	N/A
SimpleTest	Age, hyperglycemia, body mass index, platelet count, albumin, AST/ALT ratio	78%	58%	N/A
SteatoTest	FibroTest + alanine aminotransferase, body mass index, glucose, triglycerides, cholesterol, gender, age, sex	Cutoff = 0.30, 90% (vs biopsy) Cutoff = 0.30, 90% (Poynard)	Cutoff = 0.30, 54%, (vs biopsy) Cutoff = 0.30, 70% (Poynard)	0.79 0.79 (Poynard)

Table 8: The association between iron stores and type 2 diabetes mellitus

Author(s)	Year	Country / Ethnicity	Study design	Case selection / Source population	Control selection	N Cases / Controls	Age Mean (sd)	Sex (% female)	Iron Indices	Comparison	Effect size	Matching / adjustment variables
Abou-Shousha	2006	Egypt	Case-control	NA	Normal healthy volunteers	20 /10	48.5 (10.6)	Female only	Serum ferritin, sTfR	Mean difference in ferritin concentration (cases vs controls)	184.47 (5.86-363.08) ng/mL	Matching: 1,2,3,4
Ashourpour	2010	Tehran, Iran	Cross-sectional	Members of the Iranian diabetes society in Tehran	Normal healthy volunteers	54 / 53	Cases: 54 (SE: 0.45) Controls: 52.6 (SE: 0.45)		Serum ferritin	Mean difference in ferritin concentration (cases vs controls)	35.52 (6.89-64.15) ng/mL	Matching: 1,2
Elis	2004	Israel	Case-control	T2DM patients w/ severe diabetic retinopathy and DM patients w/o retinopathy	Patients free from T2DM and retiopathy	51 / 40	T2DM w/ retinopathy: 58 (3); T2DM w/o retinopathy: 63 (10); controls: 63 (14)	T2DM w/ retinopathy: 55.5; T2DM w/o retinopathy: 58.6; controls: 60	Serum ferritin, transferrin	Mean difference in ferritin concentration (cases vs controls)	26.10 (-6.50-58.70) ng/mL	Matching: 1,2,40
Elsammak	2005	Egypt	Case- control	T2DM patients	NA	22 / 18	Cases: 52.5 (7.8); controls: 51.1 (6.5)	ases: 41.0; Controls: 44.4	Serum ferritin	Mean difference in ferritin concentration (cases vs controls)	-73.00 (-5.54-100.94) ng/mL	Matching: 1,2
Ford	1999	U.S.A	Cross-sectional	NHANES III	NHANES III w/o T2DM	924 / 7,861	Non-DM: 42.1 (SE:0.4); IFG: 56.6 (SE:0.9); Newly dx DM: 59.4 (SE: 1.0); previously dx DM: 58.9 (SE: 0.8)	Non-DM: 49.1 (SE:0.7); Newly dx DM: 52.7 (SE: 4.6); previously dx DM: 43.7 (SE: 3.1)	Serum ferritin, TSAT	RR (serum ferritin)	Males: 8.96 (4.62-17.40); Females: 5.83 (2.61-13.01)	Sex stratified means; RR estimates adjusted for 1,5,6,10,22,31,32

Adapted from 395, 396

Table 8 (con't): The association between iron stores and type 2 diabetes mellitus

Author(s)	Year	Country / Ethnicity	Study design	Case selection / Source population	Control selection	N Cases / Controls	Age Mean (sd)	Sex (% female)	Iron Indices	Comparison	Effect size	Matching / adjustment variables
Ganesh	2012	India	Case-control	Attendees of the Endocrine Clinic at the M.S. Majah Hospitals	Normal healthy volunteers	30 / 30	Cases: 43.6 (9.5); controls: 41.8 (6.7)	Cases: 73.2; controls 60	Ferritin, transferrin, TSAT	Mean difference in ferritin concentration (cases vs controls)	36.19 (5.98-66.40) ng/mL	Matching: 1,2
Hughes	1998	Singapore (Chinese, Malay, Indian)	Cross-sectional	Random sample of individuals aged 40-69 yrs from Singapore gen pop	Those free from T2DM in same sample population	126 / 530	Range: 40-69	51.2	Serum ferritin	Mean difference in ferritin concentration (cases vs controls)	Males: 50.00 (20.47-79.53); Females: 51.00 (18.30-83.70)	Sex stratified means; Adjustment: 1,5
Kim	2008	U.S.A	Cross-sectional	Non-pregnant females in NHANES III w/ T2DM	Non-pregnant females in NHANES III w/o T2DM	244 / 6,015	T2DM: 46 non-T2DM: 36	Females only	Serum ferritin	Mean difference in ferritin concentration (cases vs controls)	54.00 (29.75-78.25) ng/mL	Adjustment: 1,5,7,9,10,18,19
Kim	2011	South Korea	Cross-sectional	Voluntary health check-up participants	Voluntary health check-up participants	1,054 / 11,036	Males: NFG: 50.4 (9.5); IFG: 52.2 (8.3); T2DM: 55.3 (8.6) Females: NFG: 49.5 (8.9); IFG: 53.3 (8.8); T2DM: 58.1 (8.2)	47.3	Serum ferritin	RR	1.30 (0.89-1.89)	Adjustment: 1,6,9,10,12,13,16,20,21,31
Lee	2011	South Korea	Cross-sectional	KNHANES 2008	KNHANES 2008 participants w/o/ T2DM	313 / 5,998	Males: 43.3 (SE: 0.45) Premenopausal Females: 36.9 (SE: 0.33) Postmenopausal Females: 62.6 (SE: 0.37)	57.5 (premenopausal: 33.6; postmenopausal: 23.9)	Serum ferritin	RR	Males: 1.80 (1.09-2.97) Premenopausal Females: 3.57 (1.38-9.21) Postmenopausal Females: 1.54 (0.90-2.65)	Adjustment: 1,6,9,10,22,23,24

Table 8 (con't): The association between iron stores and type 2 diabetes mellitus

Author(s)	Year	Country / Ethnicity	Study design	Case selection / Source population	Control selection	N Cases / Controls	Age Mean (sd)	Sex (% female)	Iron Indices	Comparison	Effect size	Matching / adjustment variables
Luan	2008	China	Cross-sectional	CHANES	CHANES	147 / 2,850	Males: 47.2 (14.6); Females: 46.8 (14.4)	54.0	Serum ferritin	RR	2.96 (1.53-5.72)	Adjustment: 1,2,7,9,10,11,16,20,25,39
Shi	2006	China	Cross-sectional	CHANES	CHANES participants w/o T2DM	100 / 2,722	Males: 47.2 (14.6); Females: 46.8 (14.4)	54.1	Serum ferritin	RR	1.83 (1.00-3.36)	Adjustment: 1,2,5,6,8,9,10
Skomro	2001	Canada	Case-control	T2DM patients attending hospital outpatient clinic	Non-T2DM patients attending hospital outpatient clinic	58 / 48	Cases: 57.2 (14.5); controls: 53.1 (15.2)	Cases: 50 Controls: 44.4	Serum ferritin	Mean difference in ferritin concentration (cases vs controls)	47.70 (-5.54-100.94) ng/mL	Matching: 1,2
Smotra	2008	India	Case-control	Obese patients w/ T2DM randomly selected from tertiary care hospital	Non-T2DM patients from same hospital	50 / 50	Cases: 39.1 (2.88); controls: 39 (3.10)	56	Serum ferritin	Mean difference in ferritin concentration (cases vs controls)	52.70 (31.46-73.94) ng/mL	Matching: 1,2,6
Sun	2008	China	Cross-sectional	Nutrition & Health of Aging Population in China Study participants w/ T2DM	Nutrition & Health of Aging Population in China Study participants w/o T2DM	440 / 2,725	Range: 50-70	56.2	Serum ferritin	RR	3.06 (2.20-4.27)	Adjustment: 1,2,6,7,8,9,10,11,17,22,29,30
Waheed	2009	Pakistan	Case-control	Patients w/ T2DM randomly selected from diabetes clinics	Healthy volunteers with FPG < 6 mmol/L	30 / 30	NA	NA	Serum ferritin	Mean difference in ferritin concentration (cases vs controls)	181.23 (124.89-237.57) ng/mL	Matching: 1,2

Adapted from 395, 396

Table 8 (con't): The association between iron stores and type 2 diabetes mellitus

Author(s)	Year	Country / Ethnicity	Study design	Case selection / Source population	Control selection	N Cases / Controls	Age Mean (sd)	Sex (% female)	Iron Indices	Comparison	Effect size	Matching / adjustment variables
Forouhi	2007	UK	Nested Case-control	EPIC-Norfolk Study	EPIC-Norfolk Study	360 / 758	Cases: 62.4 (8.3)	42	Serum ferritin	Mean difference in ferritin concentration (cases vs controls)	Males: 56.67 (28.74-84.60) ng/mL Females: 39.22 (13.75-64.69) ng/mL	Sex stratified means; Matching: 1,6,41,42
Jehn	2007	U.S.A	Nested Case-cohort (Follow-up time: mean 7.9 yrs)	ARIC Study	ARIC Study	599 / 690	Cases: 53.3 SE: 0.24)	Cases: 57.7	Serum ferritin	RR	0.83 (0.52-1.30)	Adjustment: 1,2,5,6,9,10,12,13,16,20,21,31,33,34,35,37,38
Jiang	2004	U.S.A	Nested case-control (Follow-up time: 10 yrs)	Nurses' Health Study	Nurses' Health Study	698 / 716	Cases: 56.5 (6.9); controls: 56.4 (6.9)	Females only	Serum ferritin	RR	2.45 (1.66-3.60)	Adjustment: 1,5,6,7,8,9,10,11,13,14,36
Le	2008	U.S.A	Cohort (Follow-up time: mean: 4.5 yrs)	ACLS participants enrolled between 1995 & 2001	ACLS participants enrolled between 1995 & 2001	220 / 5,292	Males: 48.1 (9.2); Females: 48.6 (9.2)	32	Serum ferritin	RR	1.79 (1.13-2.82)	Adjustment: 1,2,5,6,12,20,26
Rajpathak	2009	U.S.A	Nested case-control (Follow-up time: 2.8 yrs)	Placebo arm of Diabetes Prevention Program	Placebo arm of Diabetes Prevention Program	280 / 280	Cases: 50.4 (SE: 0.6); controls: 50.2 (SE: 0.6)	63.6	Serum ferritin	Mean difference in ferritin concentration (cases vs controls)	12.10 (-6.36-30.56)	Adjustment: 1,5,7,9,10,18
Salmoaa	2010	Finland	Cohort (Follow-up time: Md: 7 yrs)	Health 2000 study participants	Health 2000 study participants	179 / 4,798	Males: 52.1 (9.7) Females: 53.7 (10.5)	45.7	Serum ferritin	RR	1.52 (1.04-2.23)	Adjustment: 1,2,6,9,12,15,20,21,27,28,31

Table 9: The association between iron stores and metabolic syndrome

Author(s)	Year	Country / Ethnicity	Study design	Case selection / Source population	Control selection	N Cases / Controls	Age Mean (sd)	Sex (% female)	Iron Indices	Comparison	Effect size	Matching / adjustment variables
Lee	2014	Korea	Cross-sectional			51 / 1,299			Serum ferritin	Mean difference in ferritin concentration (SD) (cases vs controls)	41.49 (17.82) ng/mL vs. 36.67 (19.58) ng/mL	
Rajpathak	2009	U.S.A	Case-control			280 / 280			Serum ferritin	Mean difference in ferritin concentration (SD) (cases vs controls)	50.4 (0.6) ng/mL vs 50.2 (0.6) ng/mL	
Lin	2010	China	Cross-sectional			166 / 1,479			Serum ferritin	Mean difference in ferritin concentration (SD) (cases vs controls)	102.4 (65.95) ng/mL vs. 82 (59.20) ng/mL	
Gabrielson	2012	U.S.A	Cohort			38 / 87			Serum ferritin	Mean difference in ferritin concentration (SD) (cases vs controls)	260 (23) ng/mL vs. 185 (21) ng/mL	
Tang	2007	China	Case-control			60 / 63			Serum ferritin	Mean difference in ferritin concentration (SD) (cases vs controls)	199.5 (60.23) ng/mL vs. 112.45 (45.89) ng/mL	
Li	2006	China	Case-control			60 / 60			Serum ferritin	Mean difference in ferritin concentration (SD) (cases vs controls)	196.35 (72.15) µg/mL vs. 110.28 (68.32) µg/mL	
Xiao	2011	China	Case-control			87 / 102			Serum ferritin	Mean difference in ferritin concentration (SD) (cases vs controls)	243.4 (65.3) ng/mL vs. 156.5 (47.3) ng/mL	

Adapted from 11, 440

Table 9 (con't): The association between iron stores and metabolic syndrome

Author(s)	Year	Country / Ethnicity	Study design	Case selection / Source population	Control selection	N Cases / Controls	Age Mean (sd)	Sex (% female)	Iron Indices	Comparison	Effect size	Matching / adjustment variables
Iwanaga	2014	Japan	Cohort			47 / 638			Serum ferritin	Mean difference in ferritin concentration (SD) (cases vs controls)	204.7 (140.9) ng/mL vs. 96.4 (91.9) ng/mL	
Jehn	2004	U.S.A	Cross-sectional	NHANES III	NHANES III	5,949	48	52	Serum ferritin	Quartiles (Q1 vs Q4) OR	Men: 1.60 (0.92-2.77); Premenopausal Females: 2.40 (1.10-5.22); Postmenopausal Females: 2.70 (1.74-4.19)	
Bozzini	2005	Italy	Cross-sectional	Verona Heart Project	Verona Heart Project	479	58	27	Serum ferritin	OR	1.53 (1.27-1.84)	
Choi	2005	Korea	Cross-sectional	Welfare Centers of Seoul Metropolitan	Welfare Centers of Seoul Metropolitan	959	72	Females only	Serum ferritin	OR	Postmenopausal Females: 1.40 (1.23-1.59)	
Soto Gonzalez	2006	Spain	Cross-sectional	Patients of the Endocrinology & Nutrition Service of Hospital	Patients of the Endocrinology & Nutrition Service of Hospital	598	38	66	Serum ferritin	OR	2.03 (1.69-2.44)	
Vari	2007	France	Cohort	DESIR sohort	DESIR sohort	944	47	51	Serum ferritin	OR	Premenopausal Females: 1.66 (1.03-2.68); Postmenopausal Females: 1.62 (1.08-2.43)	
Shi	2008	China	Cross-sectional	CHANES 2002	CHANES 2002	2,816	Range: 40-49	54	Serum ferritin	Quartiles (Q1 vs Q4) OR	Males: 1.16 (0.73-1.84); Females: 1.66 (1.15-2.39)	
Sun	2008	China	Cross-sectional	Nutrition & Health of Aging Population in China Study participants w/ MetS	Nutrition & Health of Aging Population in China Study participants w/ MetS	3,165	58	56	Serum ferritin	Quartiles (Q1 vs Q4) OR	1.95 (1.48-2.57)	
Ryu	2008	Korea	Cross-sectional	Korean Rural GENOMIC Cohort	Korean Rural GENOMIC Cohort	1,444	58	62	Serum ferritin	Quartiles (Q1 vs Q4)	Males: 1.41 (0.74-2.68); Females: 1.61 (1.03-2.51)	
Kim	2011	Korea	Cross-sectional	Healthy volunteers	Healthy volunteers	7,253	51	47	Serum ferritin	OR	Males: 1.58 (1.06-2.36); Females: 1.07 (0.71-1.62)	
Park	2012	Korea	Cohort	Check-up of men in Health Promotion Center	Check-up of men in Health Promotion Center	13,084	44	0	Serum ferritin	OR	1.66 (1.38-2.00)	
Kang	2012	Korea	Cross-sectional	KNHANES IV	KNHANES IV	7,346	48	56	Serum ferritin	Quartiles (Q1 vs Q4)	Males: 1.46 (1.08-1.98); Females: 1.22 (0.91-1.64)	
Hamalainen	2012	Finland	Case-control	Citizens invited to health check-up 2004	Citizens invited to health check-up 2004	766	52	55	Serum ferritin	OR	Males: 1.59 (1.29-1.95); Females: 1.71 (1.40-2.09)	
Leiva	2013	Chile	Cross-sectional	Research program of Risk Factors for Cardiovascular Disease of Talca	Research program of Risk Factors for Cardiovascular Disease of Talca	155	57	69	Serum ferritin	OR	3.36 (1.75-6.45)	
Chang	2013	Taiwan	Cross-sectional	NAHSIT 2005-2008	NAHSIT 2005-2008	2,654	55	57	Serum ferritin	OR	1.72 (1.21-2.45)	
Li	2013	China	Cross-sectional	CHANES	CHANES	8,441	51	53	Serum ferritin	Quartiles (Q1 vs Q4)	Males: 4.05 (3.19-5.14); Females: 2.34 (1.84-2.97)	

Adapted from 11, 440

Table 10: The association between iron stores and non-alcoholic fatty liver disease

Author(s)	Year	Country / Ethnicity	Study design	Case selection / Source population	Follow-up Period (months)	N Cases / Controls	Age Mean (sd)	Sex (% female)	Definition of Phlebotomy & Iron Indices	Comparison	Effect size	Matching / adjustment variables
Valenti	2007	Italy	Non-randomized controlled	3 liver units in northern Italy	12	64 / 64	Phlebotomy group: Controls:	17	Removing 350cc of blood every 10-15 days according to baseline Hb until ferritin concentration was < 80ng/mL in the presence of low TSAT	HOMA-IR Mean Difference between groups after phlebotomy	1.73 (1.27-2.19)	
Valenti	2011	Italy	Non-randomized controlled	3 Italian centers across an 18 month period	6-8	79 / 119	Phlebotomy group: Controls:	16.7	Weekly or fortnightly removal of 350cc of blood according to baseline Hb concentration until ferritin concentration was < 80ng/mL in the presence of low TSAT	HOMA-IR Mean Difference between groups after phlebotomy	1.14 (0.57-1.71)	
Valenti	2014	Italy	Randomized controlled		24	21 / 17	Phlebotomy group: Controls:	10.5	Removing 350cc of blood every 10-15 days according to baseline Hb until ferritin concentration was < 30ng/mL or TSAT < 25%	HOMA-IR Mean Difference between groups after phlebotomy	0.30 (-0.45-1.05)	
Adams	2016	Australia	Randomized controlled		6	33 / 41	Phlebotomy group: Controls:	40.5	Removing 200-300mL of blood every 2-3 weeks according to baseline Hb until ferritin concentration was < 45ng/mL	HOMA-IR Mean Difference between groups after phlebotomy	0.20 (0.02-0.38)	
Perez-Aguilar	2004	Spain	Genetic association	Histologically confirmed NAFLD	Healthy volunteers	22 / 21			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NAFLD patients vs. controls	0.67 (0.39-1.16)	
Lin	2005	Taiwan	Genetic association	Histologically confirmed NAFLD	Healthy volunteers	33 / 125			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NAFLD patients vs. controls	0.96 (0.16-5.67)	
Simsek	2006	Turkey	Genetic association	Histologically confirmed NAFLD	Healthy volunteers	30 / 2,677			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NAFLD patients vs. controls	2.29 (1.12-4.70)	*
Lee	2010	Korea	Genetic association	Histologically confirmed NAFLD	Healthy volunteers	125 / 221			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NAFLD patients vs. controls	1.54 (1.09-2.20)	*
Floreani	2007	Italy	Genetic association	Histologically confirmed NAFLD	Healthy volunteers	171 / 205			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NAFLD patients vs. controls	1.03 (0.82-1.31)	
Aigner	2008	Austria	Genetic association	Histologically confirmed NAFLD	Liver biopsies with unexplained pathology	61 / 20			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NAFLD patients vs. controls	0.95 (0.80-1.12)	

Adapted from 468. Any HFE mutation defined as: C282Y/C282Y; C282Y/H63D; C282Y/WT; H63D/H63D; H63D/WT

Table 10: The association between iron stores and non-alcoholic fatty liver disease

Author(s)	Year	Country / Ethnicity	Study design	Case selection / Source population	Control selection	N Cases / Controls	Age Mean (sd)	Sex (% female)	Iron Indices	Comparison	Effect size	Matching / adjustment variables
Venat	2003	France	Genetic association	Histologically confirmed NAFLD	Healthy volunteers	12 / 9			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NAFLD patients vs. controls	1.43 (0.71-2.88)	
Pucelikova	2004	Czech Republic	Genetic association	Histologically confirmed NAFLD	Healthy volunteers	41 / 257			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NAFLD patients vs. controls	1.52 (0.81-2.84)	
Begianesi	2004	Italy	Genetic association	Histologically confirmed NAFLD	Blood donors	210 / 204			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NAFLD patients vs. controls	1.09 (0.89-1.34)	
Mendler	1999	France	Genetic association	Histologically confirmed NAFLD	Healthy volunteers	86 / 75			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NAFLD patients vs. controls	0.72 (0.54-0.95)	*

Adapted from 468. Any HFE mutation defined as: C282Y/C282Y; C282Y/H63D; C282Y/WT; H63D/H63D; H63D/WT

Table 10: The association between iron stores and non-alcoholic steatohepatitis

Author(s)	Year	Country / Ethnicity	Study design	Case selection / Source population	Control selection	N Cases / Controls	Age Mean (sd)	Sex (% female)	Iron Indices	Comparison	Effect size	Matching / adjustment variables
George	1998	Australia	Genetic association	Histologically confirmed NASH	Healthy volunteers	51 / 2465			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NASH patients vs. controls	1.54 (0.89-2.65)	
Bonkovsky	2000	U.S.A	Genetic association	Histologically confirmed NASH	Historic controls	57 / 348			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NASH patients vs. controls	2.36 (1.42-3.92)	*
Chitturi	2002	Australia	Genetic association	Histologically confirmed NASH	Blood donors	42 / 127			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NASH patients vs. controls	1.09 (0.64-1.87)	
Valenti	2009	Italy	Genetic association	Histologically confirmed NASH	Blood donors	594 / 184			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NASH patients vs. controls	1.00 (0.92-1.09)	
Zamin	2006	Brazil	Genetic association	Histologically confirmed NASH	Healthy volunteers	29 / 20			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NASH patients vs. controls	1.28 (0.80-2.05)	
Neri	2007	Italy	Genetic association	Histologically confirmed NASH	Blood donors	272 / 430			Any iron-loading HFE mutation	Odds (OR) of any HFE mutation in NASH patients vs. controls	0.71 (0.57-0.89)	*

Adapted from 468. Any HFE mutation defined as: C282Y/C282Y; C282Y/H63D; C282Y/WT; H63D/H63D; H63D/WT

Table 11: Matching indices for study summaries	
Index	Variable
1	Age
2	Sex
3	Height
4	Weight
5	Ethnicity
6	BMI
7	Family history of diabetes
8	Physical activity
9	Smoking
10	Alcohol consumption
11	Dietary factors
12	Cholesterol
13	Menopausal status
14	Fasting status
15	Blood glucose
16	Waist circumference/central obesity
17	Leg length
18	Parity
19	SES
20	Hypertension
21	Triglycerides
22	Education
23	AST
24	ALT
25	Sedentary time
26	Obesity
27	Medication used to treat hypertension
28	History of cardiovascular disease
29	Geographic location
30	Use of iron supplements
31	Inflammatory factors (e.g., CRP)
32	Examination session attended
33	Fasting insulin concentration
34	Study center
35	Fasting glucose concentration
36	Glycemic load
37	Glycated hemoglobin
38	sTfR
39	Abnormal blood lipids
40	Hemoglobin concentration
41	General practice
42	Recruitment date

Adapted from 395

Table 12: Odds of metabolic dysfunction (continuously-scaled predictors)									
		Fe_{COOK}				TSAT			
Outcome	Type of Model & Covariates	Unadjusted OR	Unadjusted P-value	Adjusted OR	Adjusted P-value	Unadjusted OR	Unadjusted P-value	Adjusted OR	Adjusted P-value
T2DM	Basic Model	1.03	0.15	-	-	0.97	0.08		
	Age	-	-	1.01	0.43			0.97	0.08
	Ethnicity	-	-	1.05	0.05			0.97	0.17
	Education	-	-	1.03	0.16			0.98	0.08
	BMI	-	-	1.03	0.24			0.97	0.84
	Age at menarche	-	-	1.03	0.25			0.97	0.09
	OC use	-	-	1.02	0.40			0.97	0.09
	Parity	-	-	1.03	0.21			0.97	0.13
	Menopausal Status	-	-	1.00	0.94			0.97	0.08
	Alcohol Consumption	-	-	1.04	0.12			0.97	0.09
	Smoking History	-	-	1.03	0.17			0.97	0.07
	Physical Activity	-	-	1.03	0.16			0.97	0.08
	C-reactive protein	-	-	1.02	0.37			0.99	0.43
	Full model	-	-	**1.02**	**0.53**			**1.00**	**0.91**
Metabolic Syndrome	Basic Model	1.01	0.23	-	-	0.97	<0.0001		
	Age	-	-	1.00	0.92			0.96	<0.0001
	Ethnicity	-	-	1.01	0.13			0.96	<0.0001
	Education	-	-	1.01	0.35			0.96	<0.0001
	BMI	-	-	1.01	0.53			0.99	0.21
	Age at menarche	-	-	1.01	0.64			0.96	<0.0001
	OC use	-	-	1.00	0.86			0.96	<0.0001
	Parity	-	-	1.01	0.54			0.97	<0.0001
	Menopausal Status	-	-	0.99	0.36			0.96	<0.0001
	Alcohol Consumption	-	-	1.01	0.36			0.96	<0.0001
	Smoking History	-	-	1.01	0.31			0.96	<0.0001
	Physical Activity	-	-	1.01	0.24			0.96	<0.0001
	C-reactive protein	-	-	1.00	0.73			0.97	0.0003
	Full model	-	-	**0.99**	**0.28**			**0.98**	**0.14**

Table 12 (continued): Odds of metabolic dysfunction (continuously-scaled predictors)									
		Fe_{COOK}				TSAT			
Outcome	Type of Model & Covariates	Unadjusted OR	Unadjusted P-value	Adjusted OR	Adjusted P-value	Unadjusted OR	Unadjusted P-value	Adjusted OR	Adjusted P-value
NALFD	Basic Model	1.04	0.04	-	-	0.96	<0.0001		
	Age	-	-	1.02	0.11			0.96	<0.0001
	Ethnicity	-	-	1.04	0.02			0.96	<0.0001
	Education	-	-	1.04	0.04			0.96	<0.0001
	BMI	-	-	1.01	0.48			0.98	0.13
	Age at menarche	-	-	1.04	0.09			0.96	<0.0001
	OC use	-	-	1.03	0.11			0.96	<0.0001
	Parity	-	-	1.03	0.09			0.96	<0.0001
	Menopausal Status	-	-	1.03	0.21			0.96	<0.0001
	Alcohol Consumption	-	-	1.02	0.31			0.95	<0.0001
	Smoking History	-	-	1.02	0.35			0.95	<0.0001
	Physical Activity	-	-	1.02	0.31			0.95	<0.0001
	C-reactive protein	-	-	1.02	0.22			0.97	0.001
	Full model	-	-	**1.00**	**0.99**			**0.99**	**0.26**
Insulin Resistance	Basic Model	1.04	0.05	-	-	0.97	0.01		
	Age	-	-	1.04	0.04			0.98	0.03
	Ethnicity	-	-	1.05	0.01			0.98	0.06
	Education	-	-	1.04	0.02			0.98	0.02
	BMI	-	-	1.03	0.20			0.99	0.51
	Age at menarche	-	-	1.04	0.04			0.98	0.06
	OC use	-	-	1.04	0.03			0.97	0.05
	Parity	-	-	1.04	0.04			0.97	0.03
	Menopausal Status	-	-	1.04	0.07			0.97	0.04
	Alcohol Consumption	-	-	1.03	0.12			0.97	0.01
	Smoking History	-	-	1.03	0.17			0.97	0.01
	Physical Activity	-	-	1.03	0.22			0.96	0.002
	C-reactive protein	-	-	1.02	0.27			0.98	0.16
	Full model	-	-	**1.04**	**0.07**			**1.00**	**0.93**

Table 13: Odds of metabolic dysfunction: highest tertile versus lowest tertile							
		Fe_{COOK}			TSAT		
Outcome	Categorization	OR	95%CI	P_{trend}	OR	95%CI	P_{trend}
T2DM	All females	1.30	0.90-1.89	0.17	0.48	0.26-0.86	0.01
	White	1.01	0.57-2.19	0.75	0.41	0.17-1.04	0.06
	Hispanic	1.52	0.87-2.64	0.14	0.70	0.30-1.66	0.42
	Black	2.52	1.39-4.57	0.002	0.74	0.23-2.40	0.61
Metabolic Syndrome	All females	1.14	0.94-1.39	0.18	0.47	0.31-0.71	0.0003
	White	1.08	0.82-1.41	0.61	0.44	0.25-0.77	0.004
	Hispanic	1.28	0.90-1.81	0.005	0.54	0.25-1.15	0.11
	Black	1.57	1.15-2.16	0.02	0.50	0.22-1.11	0.09
NAFLD	All females	1.35	1.00-1.81	0.05	0.41	0.28-0.61	<0.0001
	White	1.52	1.00-2.31	0.05	0.39	0.22-0.69	0.001
	Hispanic	1.10	0.69-1.75	0.69	0.40	0.17-0.90	0.02
	Black	1.62	0.88-3.01	0.12	0.69	0.29-1.64	0.40
Insulin Resistance	All females	1.46	1.03-2.06	0.03	0.50	0.28-0.90	0.02
	White	2.59	1.45-4.65	0.005	0.56	0.23-1.40	0.28
	Hispanic	1.04	0.60-1.78	0.58	0.48	0.17-1.34	0.19
	Black	1.25	0.80-1.96	0.47	0.74	0.30-1.83	0.77

Table 14: Association between highest vs. lowest Fe_{COOK} tertile, postmenopausal status and metabolic dysfunction			
Outcome	OR	95%CI	P_{trend}
T2DM	1.12	0.15-8.62	0.91
Metabolic Syndrome	1.10	0.46-2.62	0.83
NAFLD	1.76	0.41-7.46	0.44
Insulin Resistance	2.24	0.30-16.58	0.43

Table 15: Association between highest vs. lowest TSAT tertile, postmenopausal status and metabolic dysfunction			
Outcome	OR	95%CI	P_{trend}
T2DM	1.00	0.16-6.31	0.99
Metabolic Syndrome	0.97	0.37-2.48	0.94
NAFLD	2.93	0.39-22.12	0.30
Insulin Resistance	1.64	0.18-15.39	0.66

Figure 2

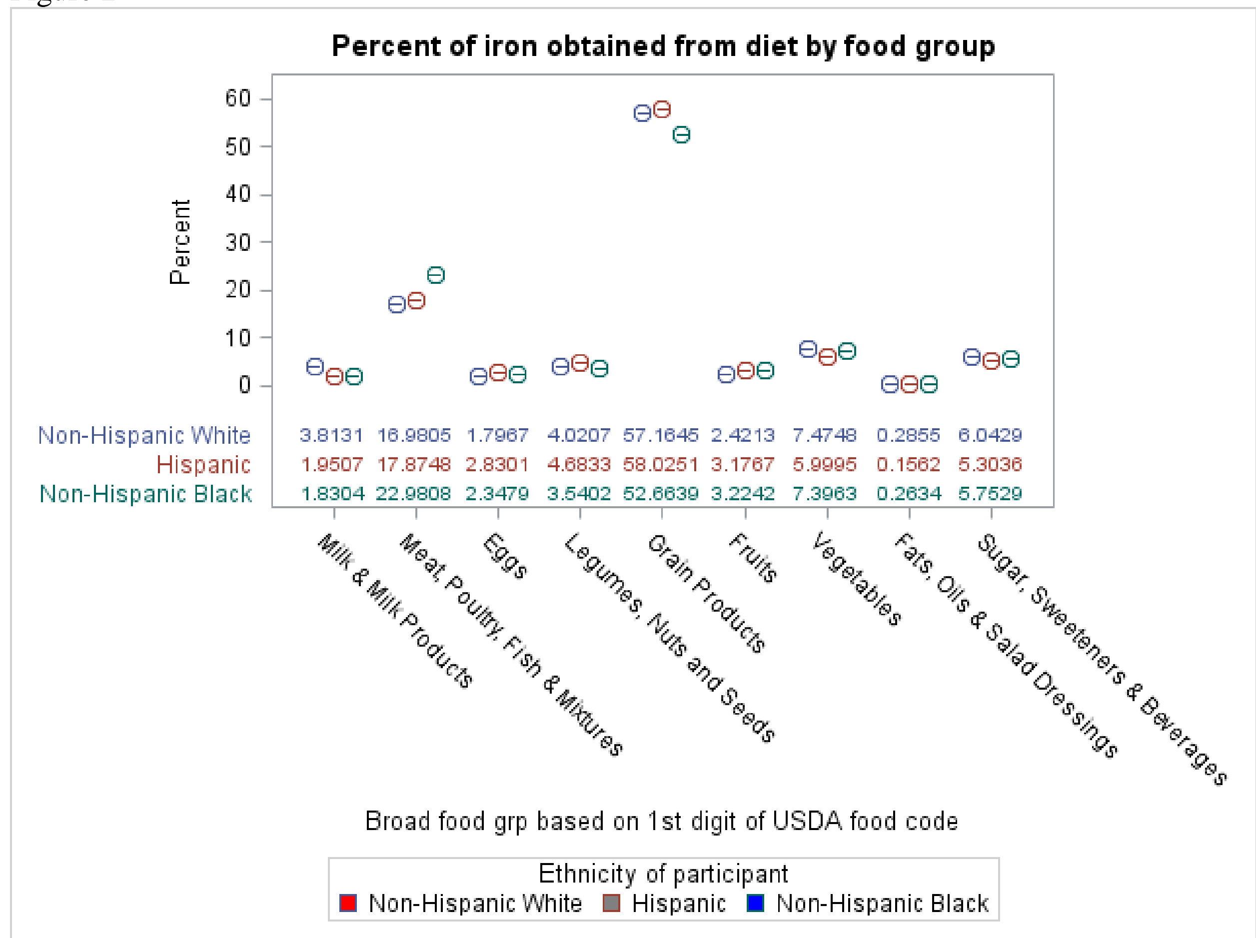
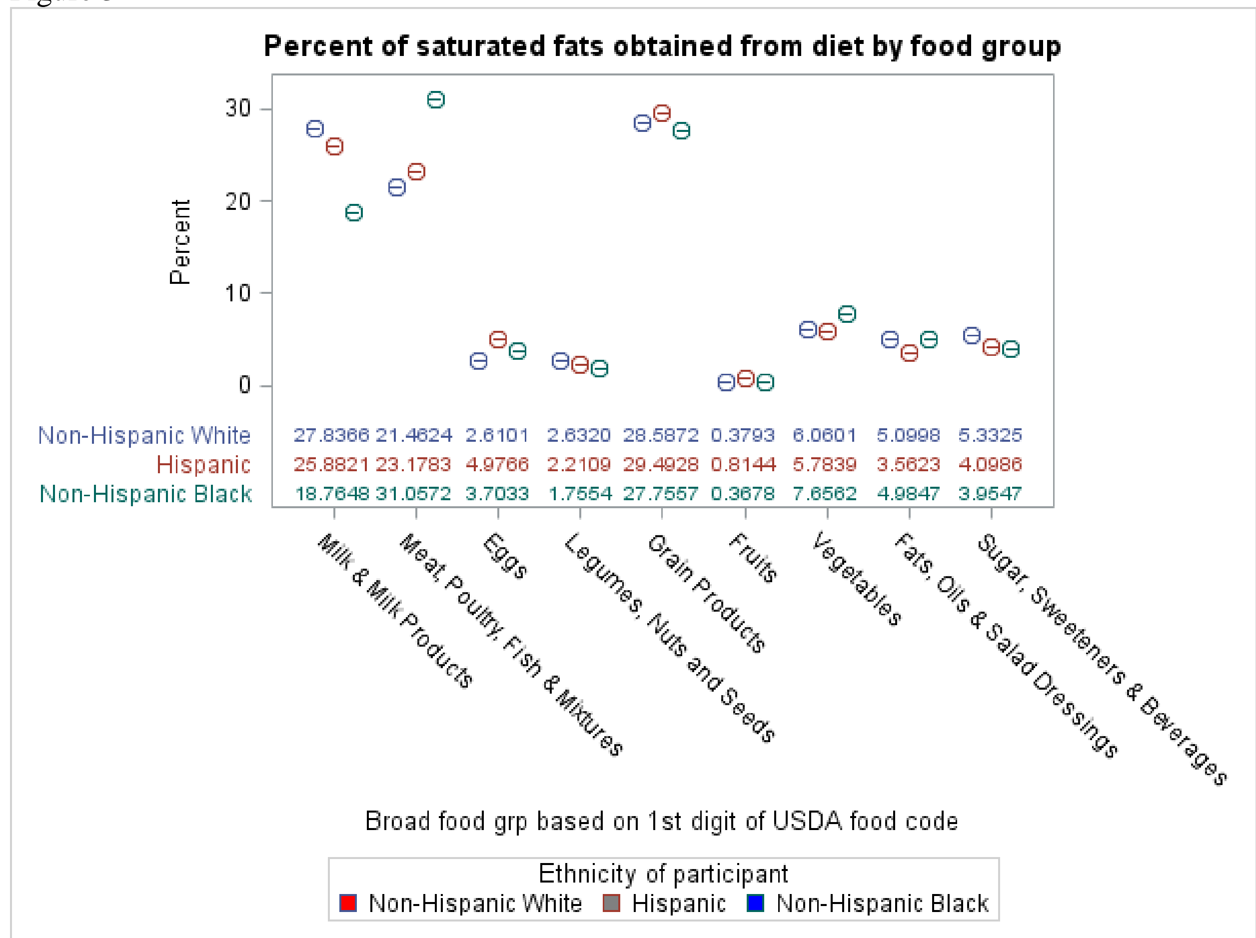


Figure 3



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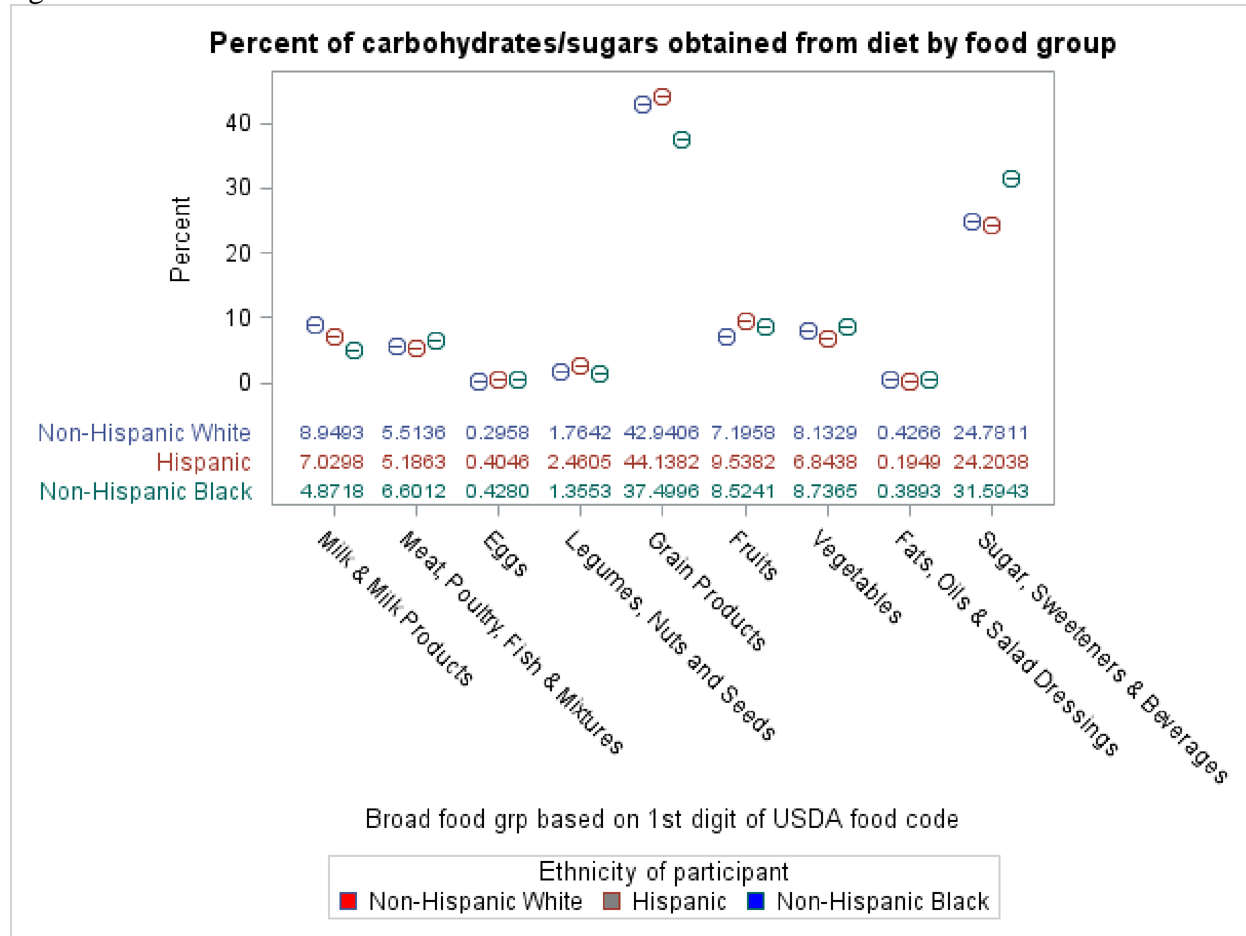

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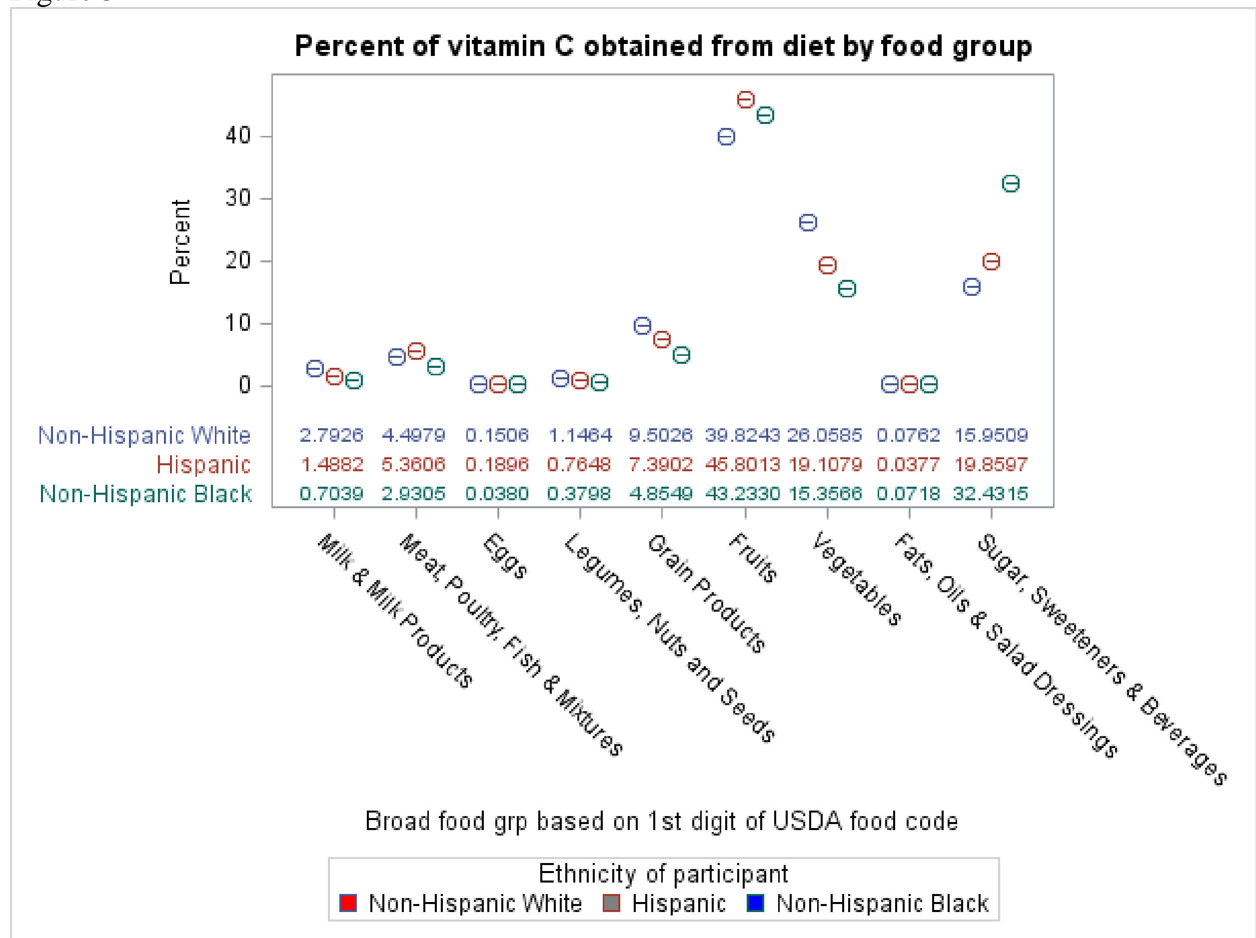

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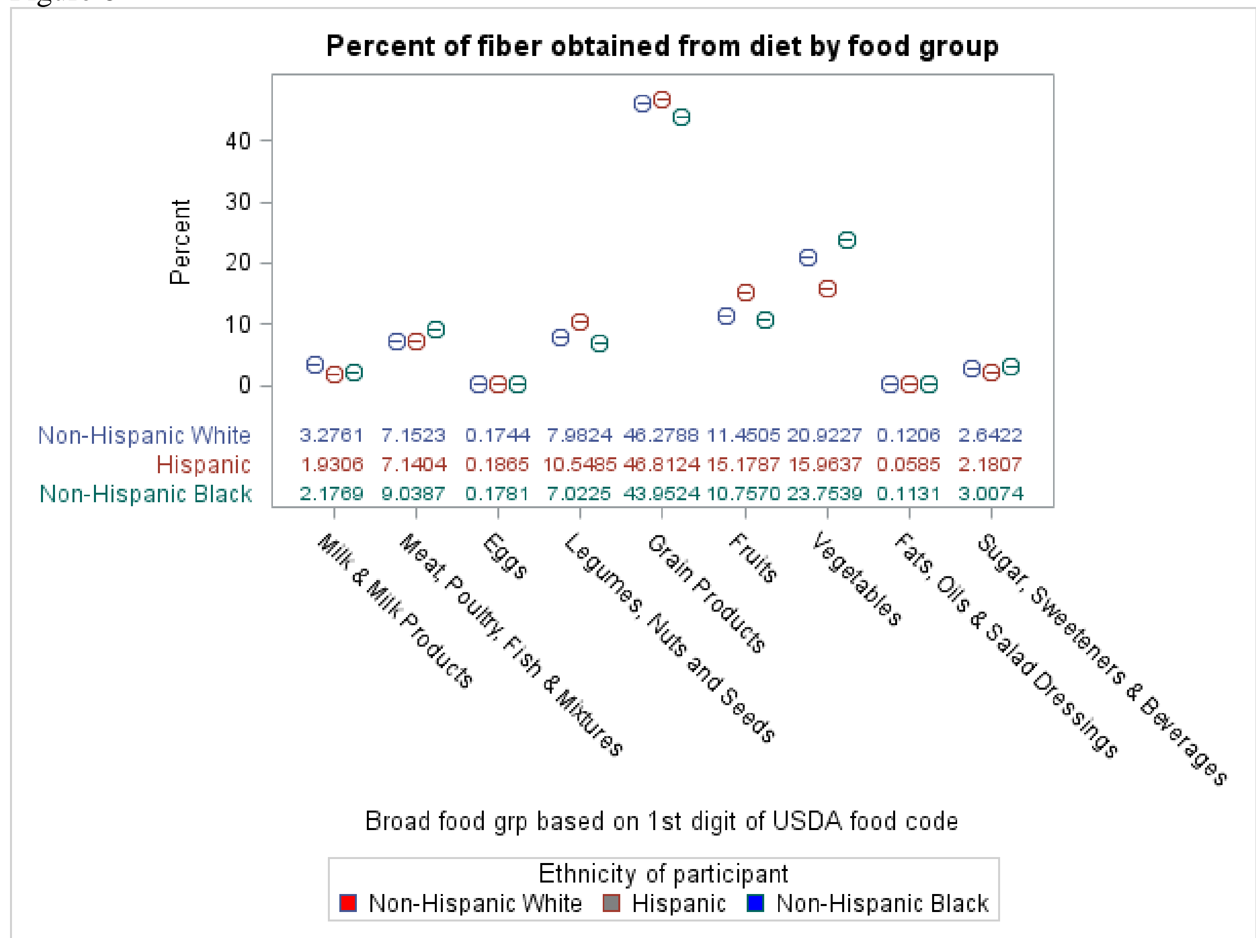

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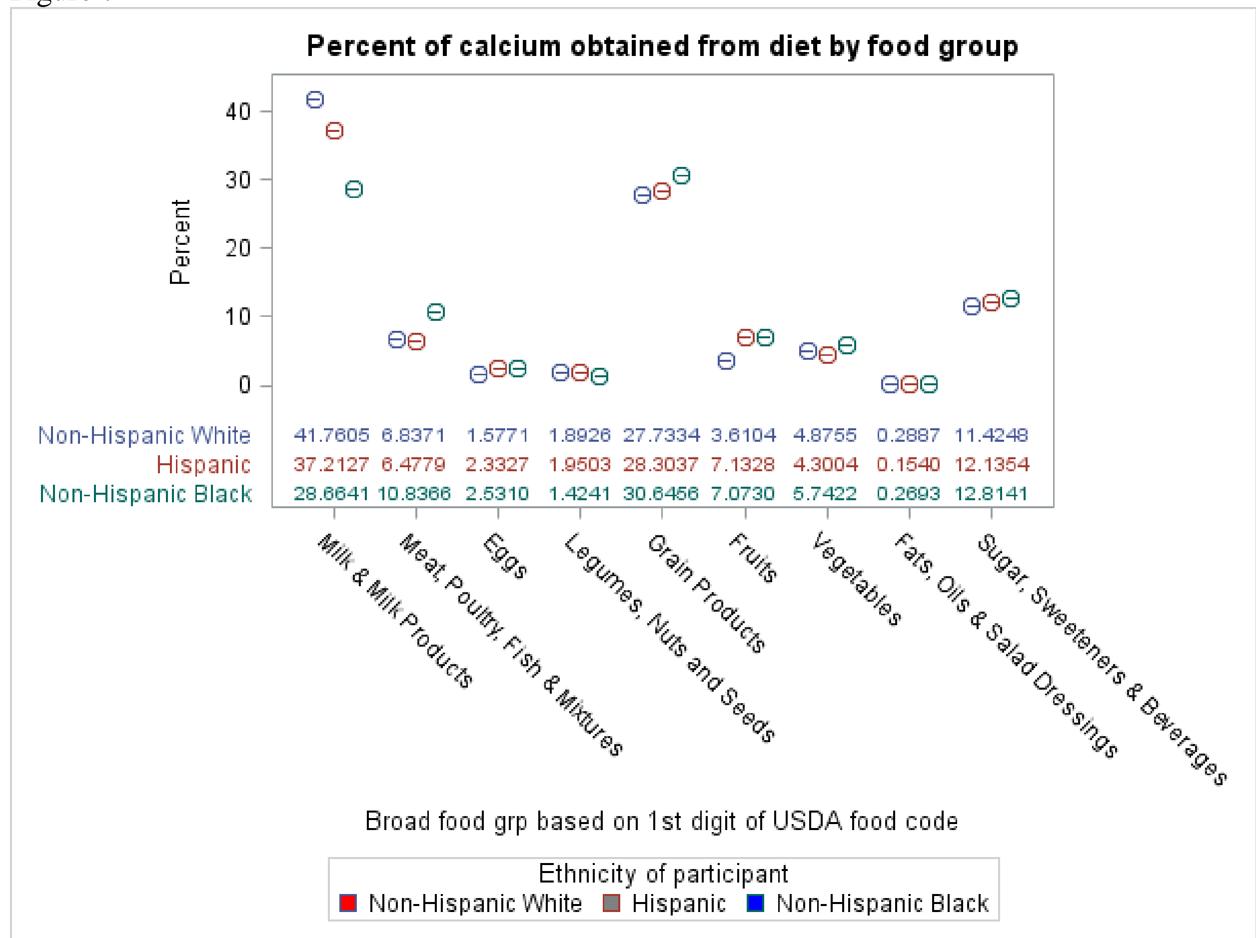

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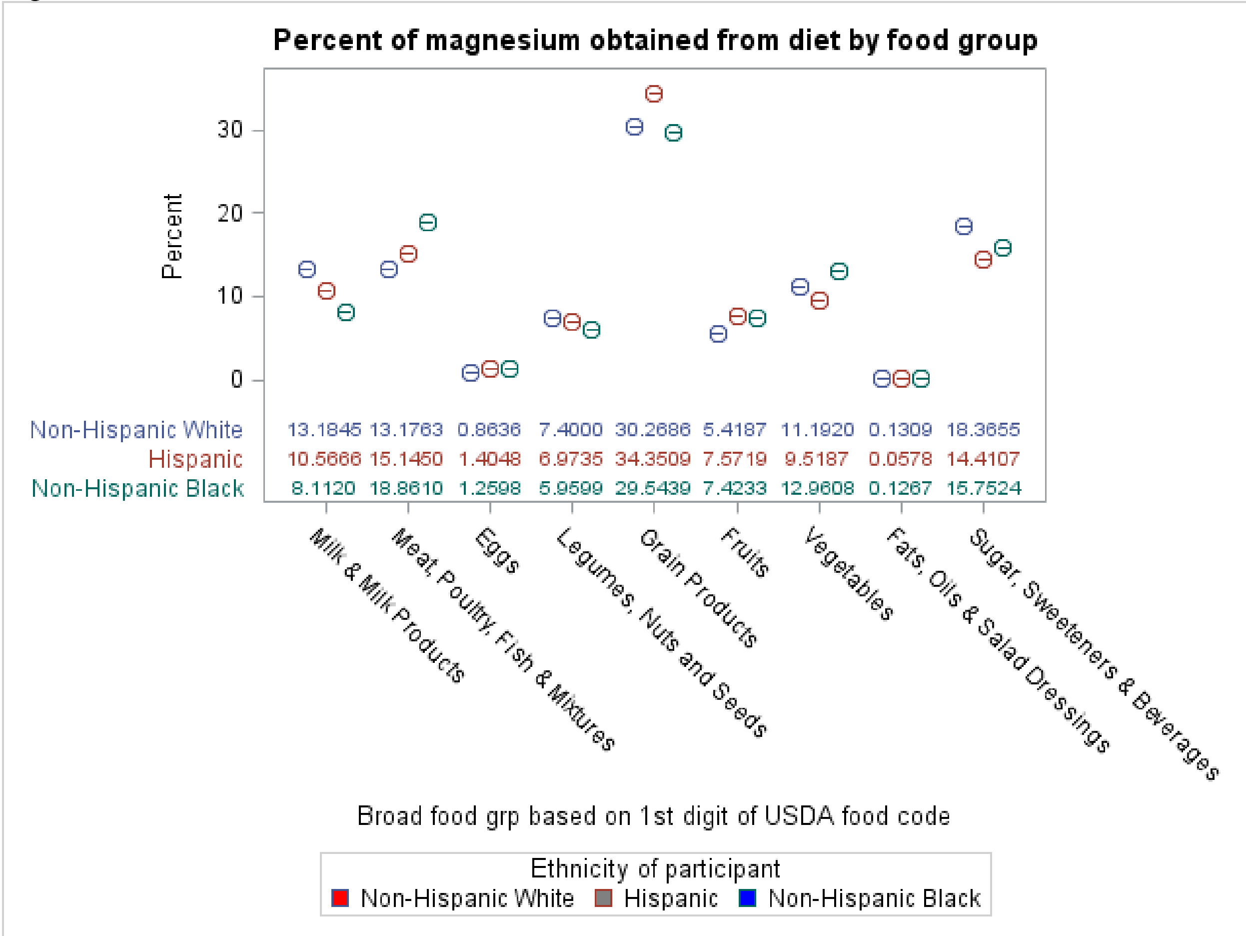

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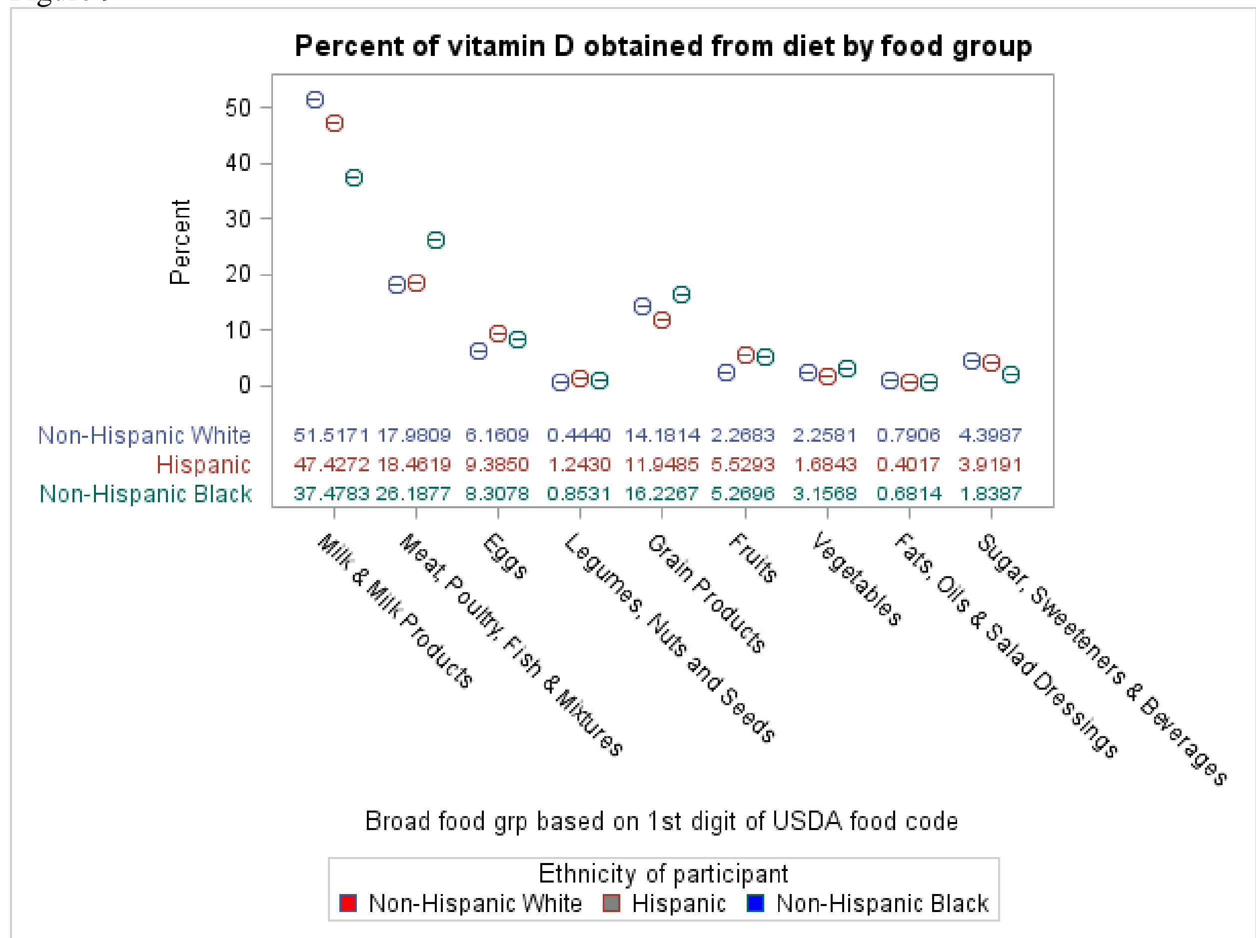

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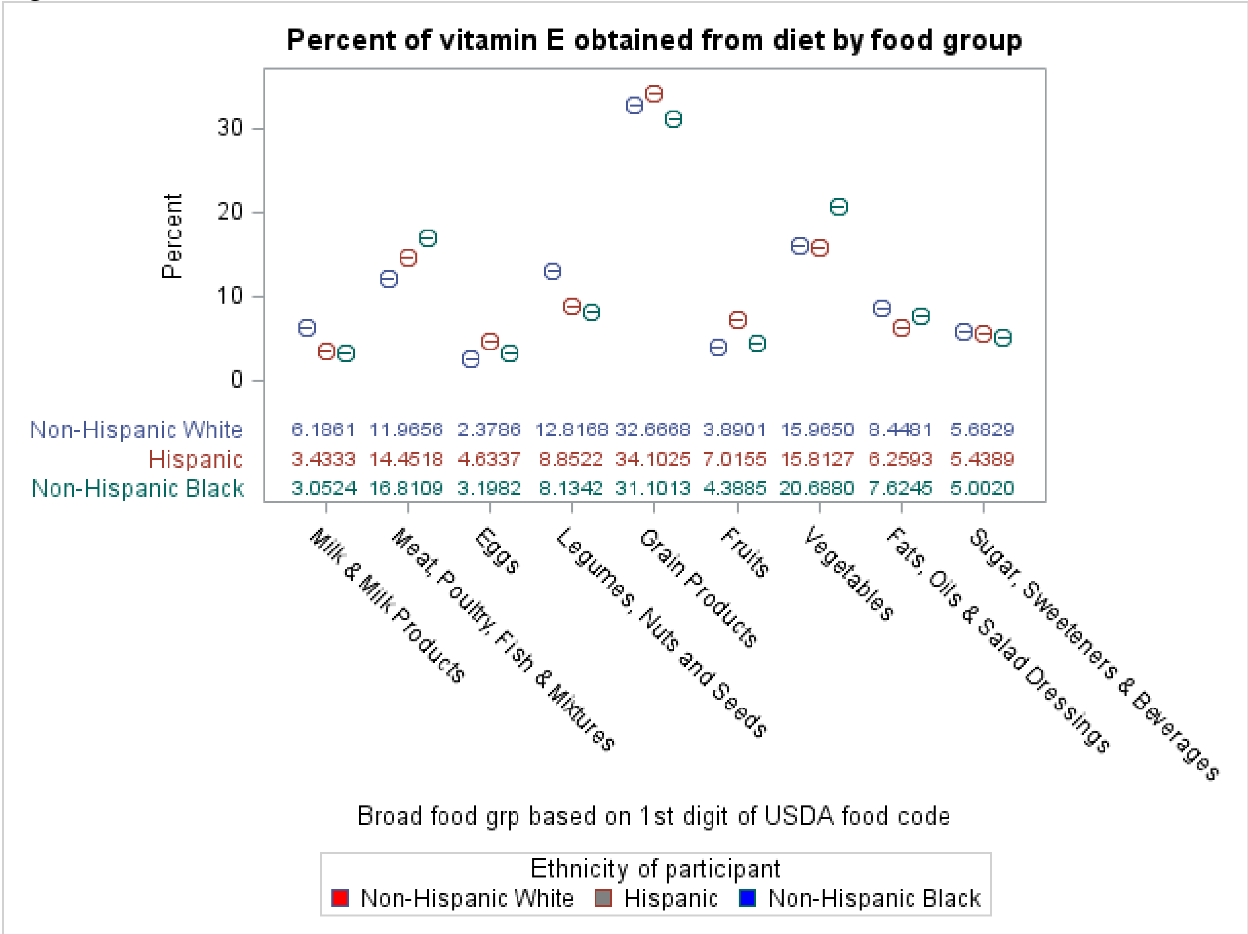

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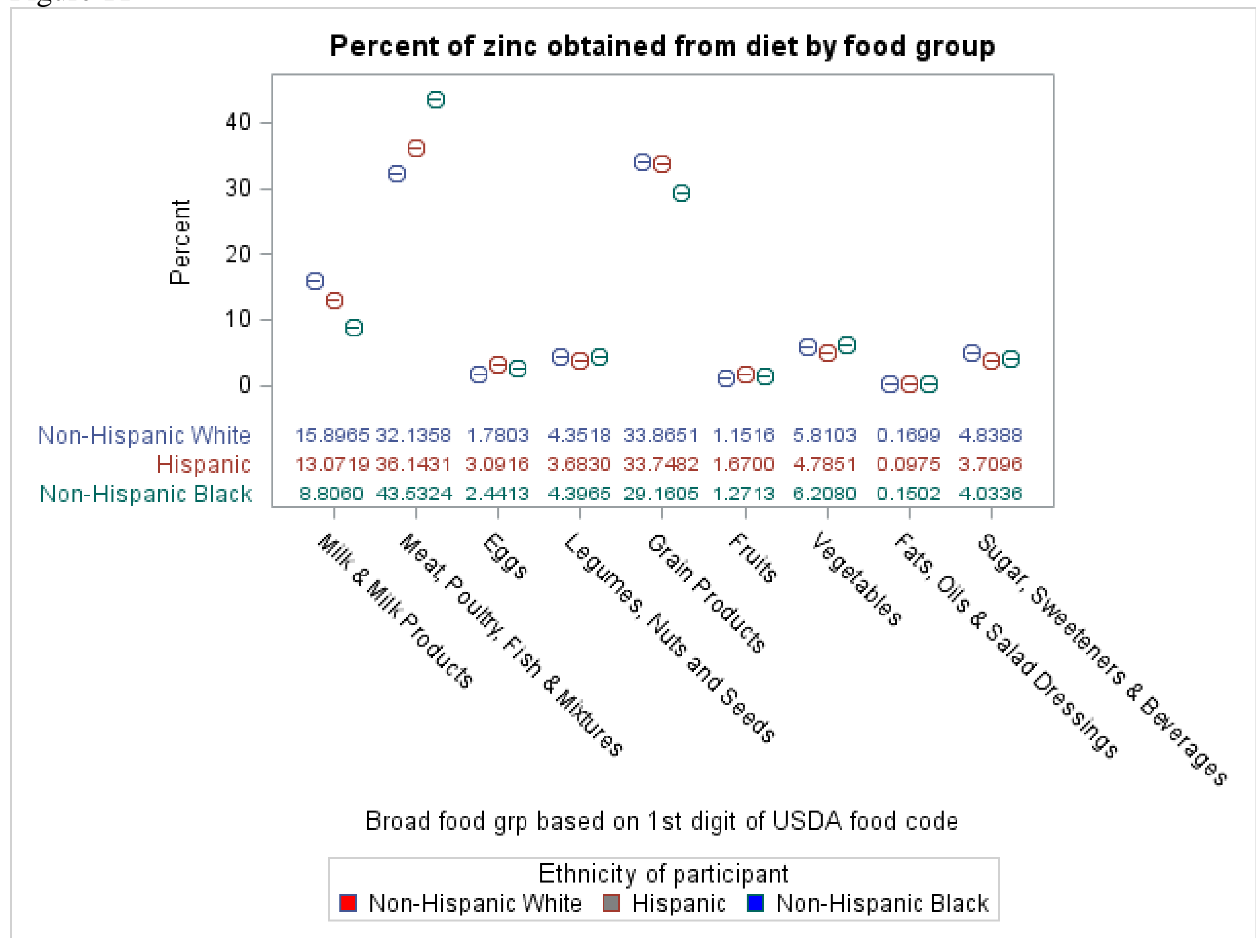

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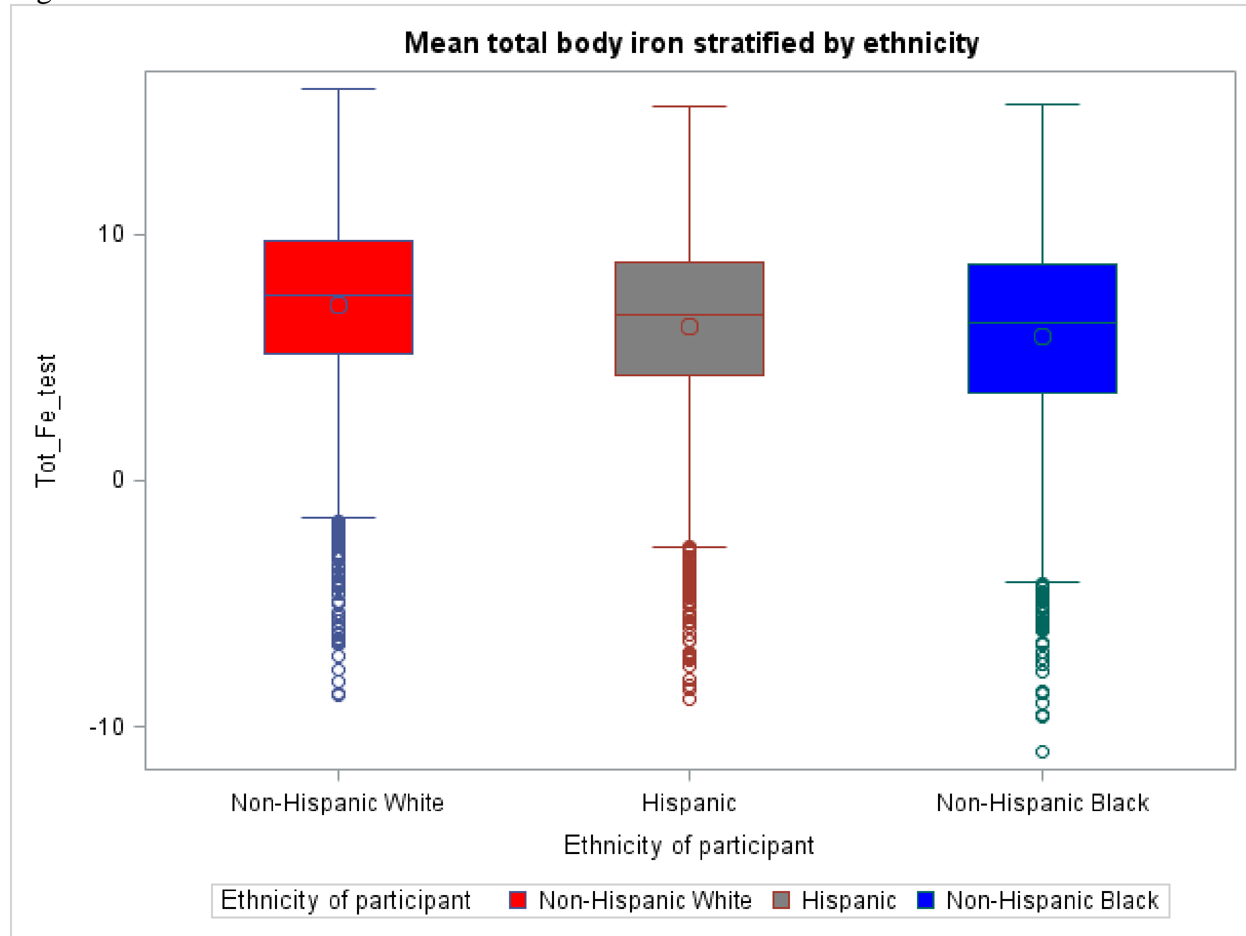

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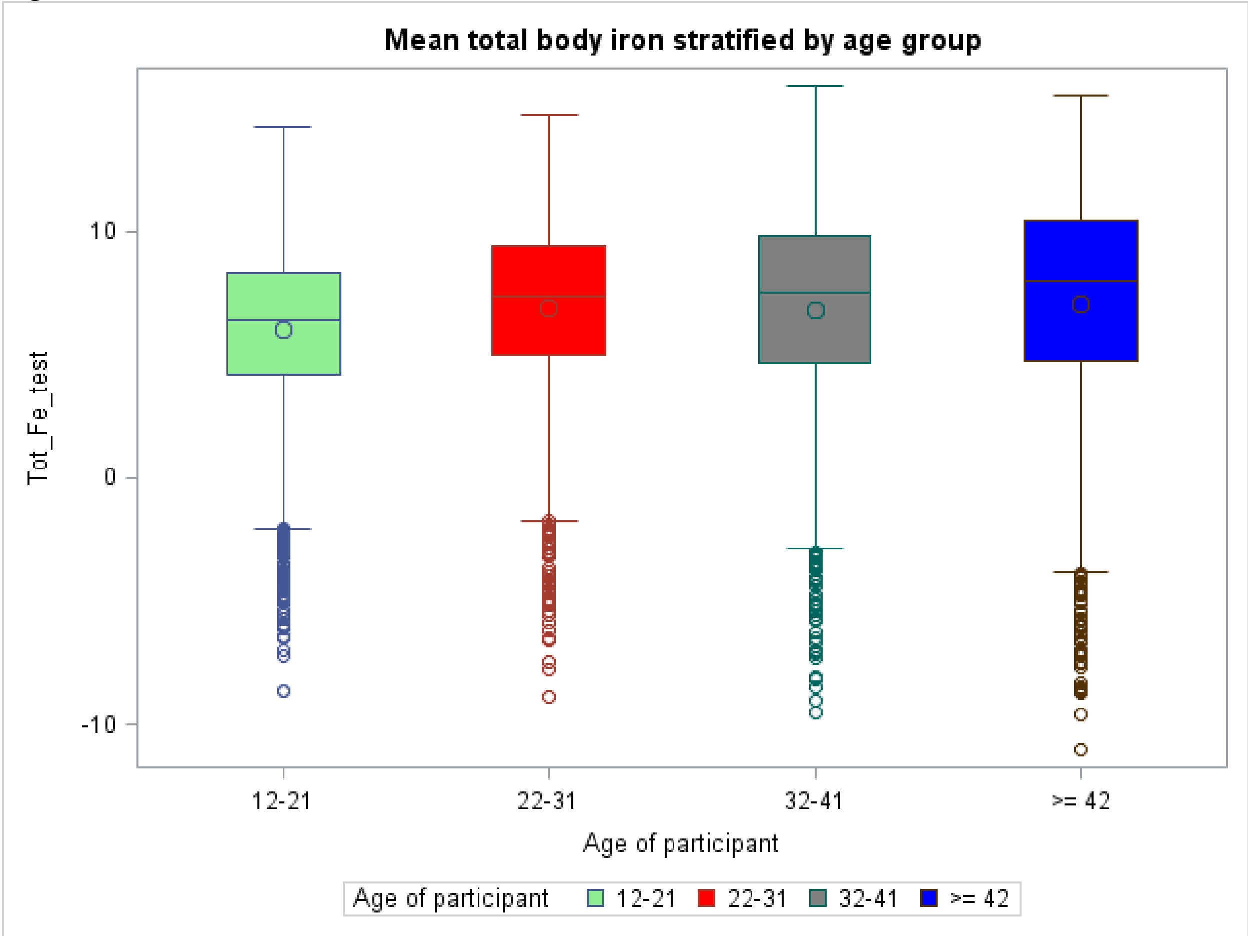

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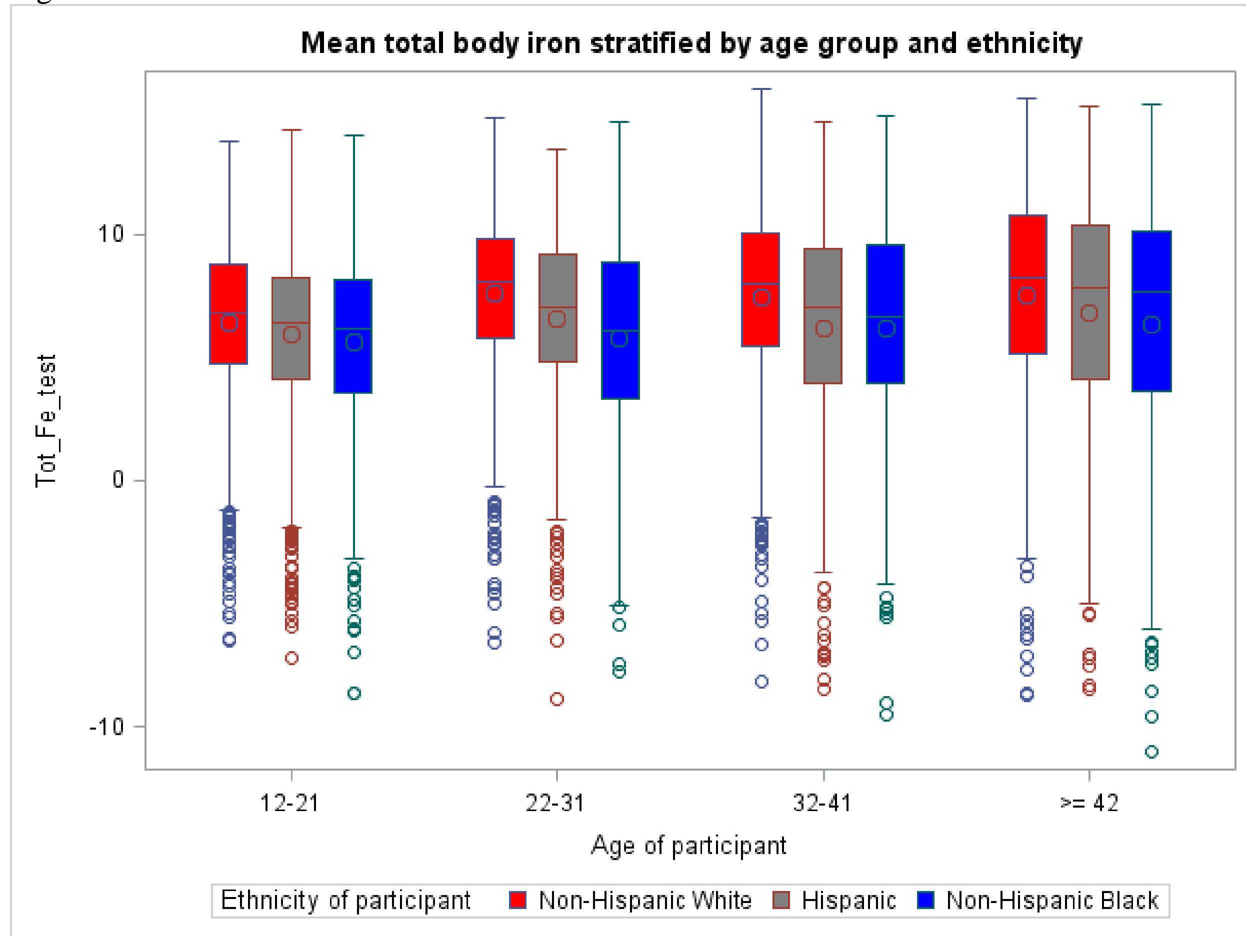
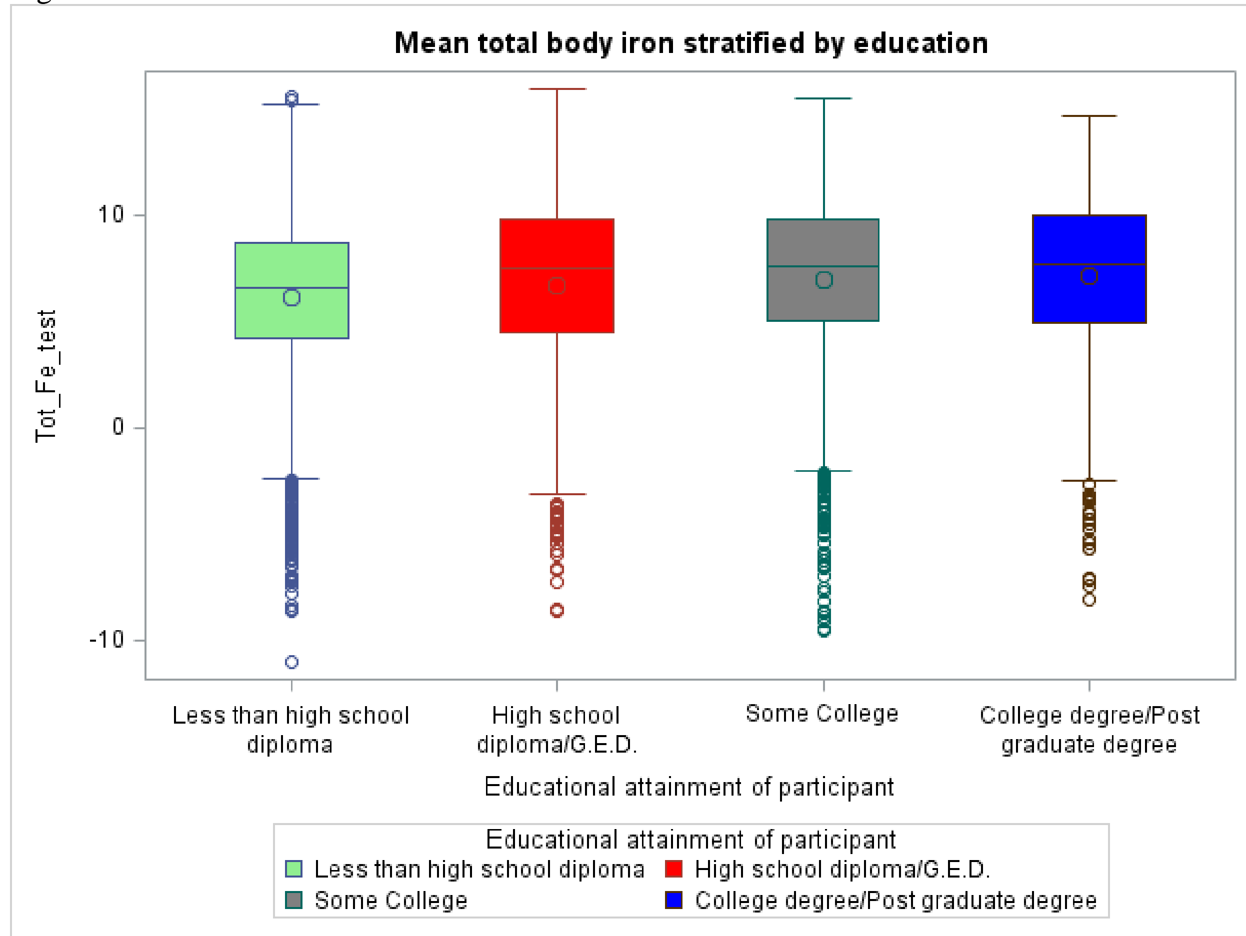


Figure 15



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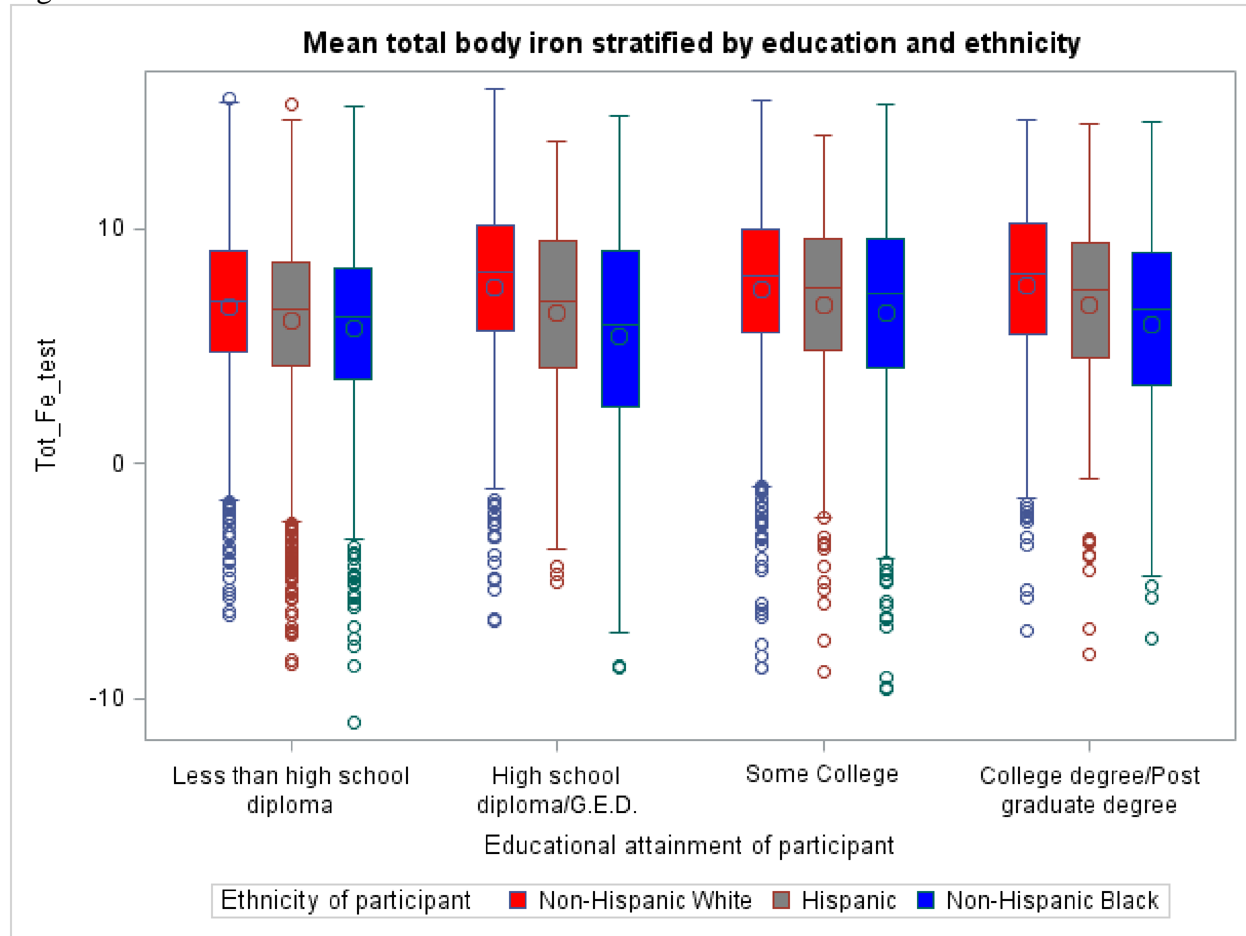

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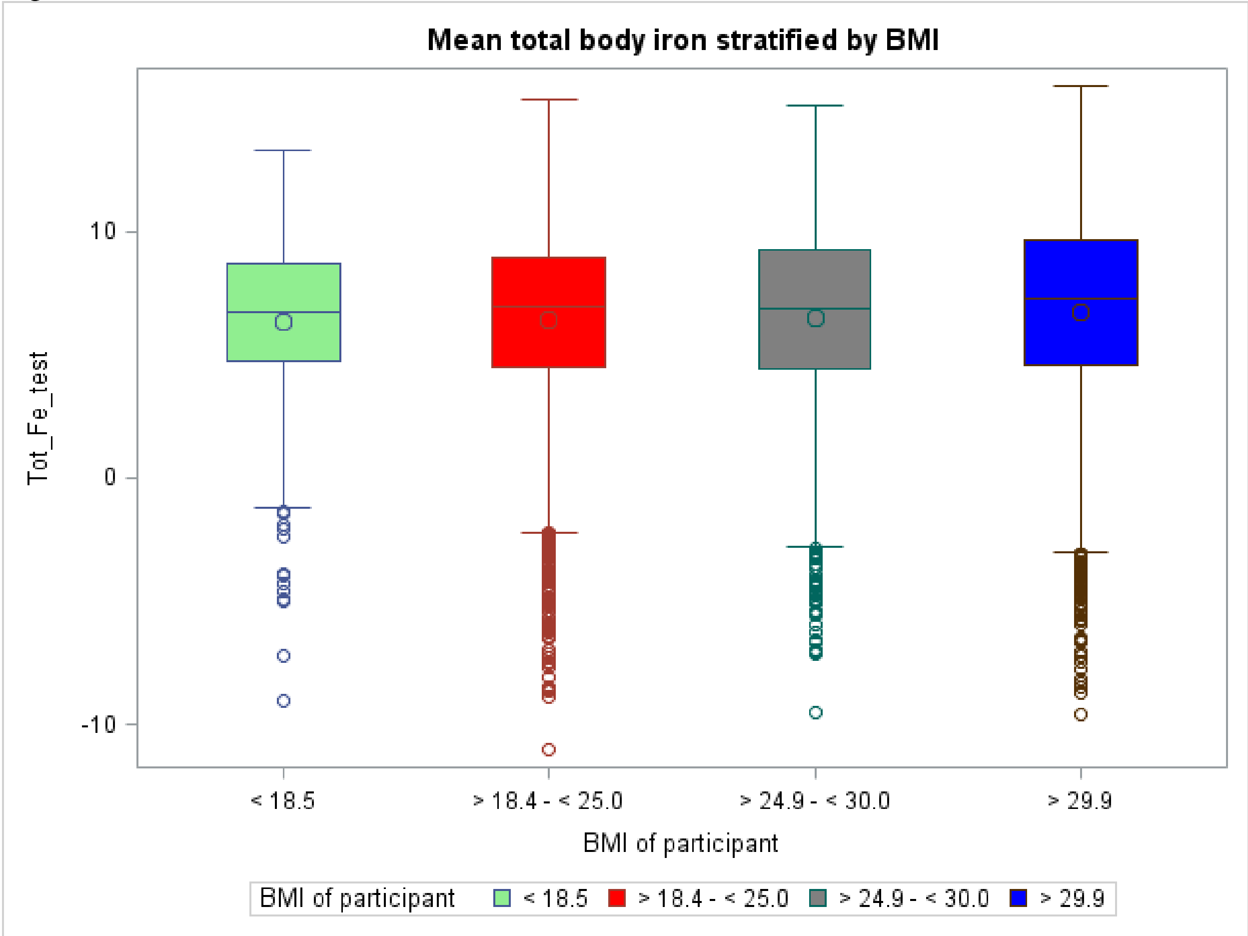

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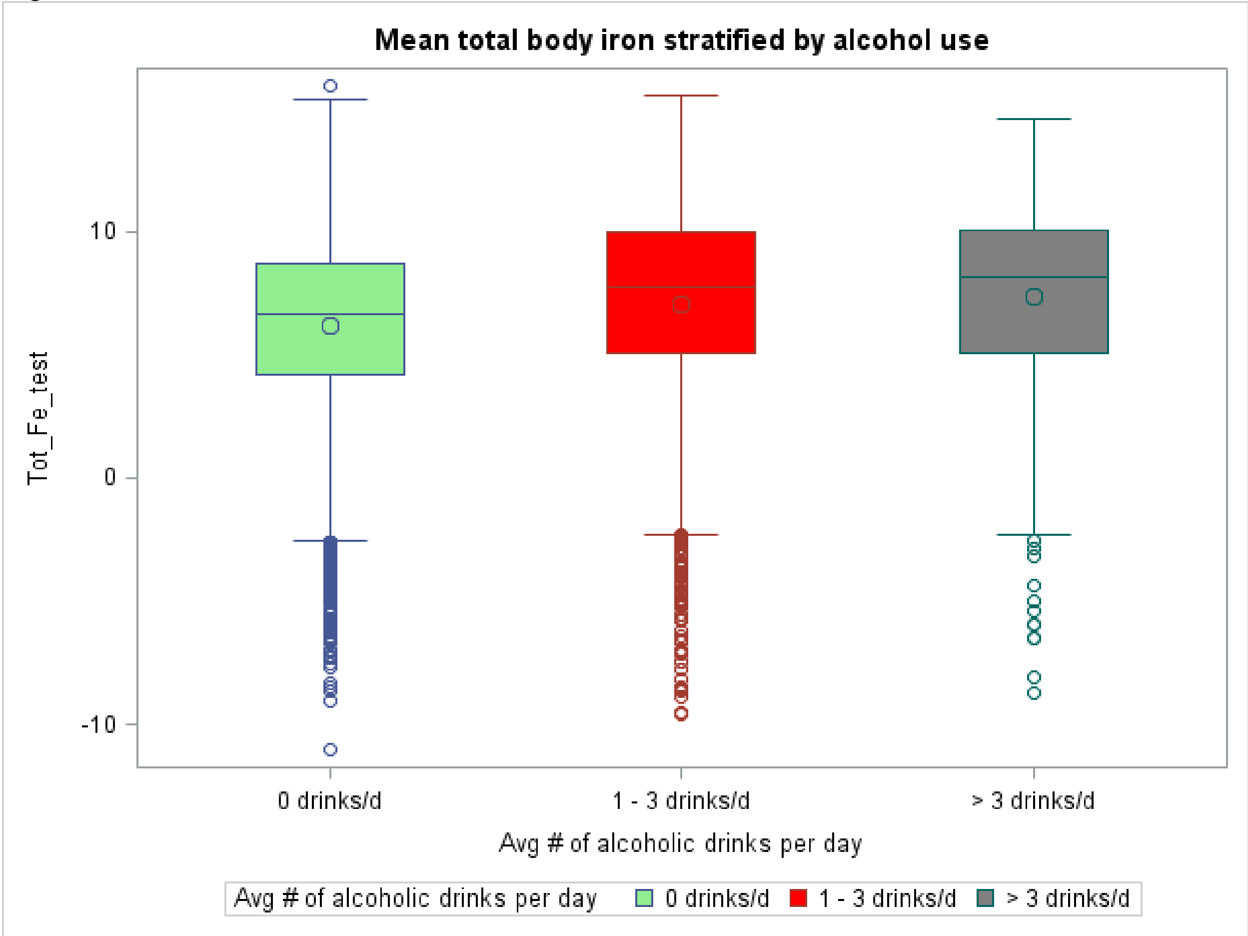

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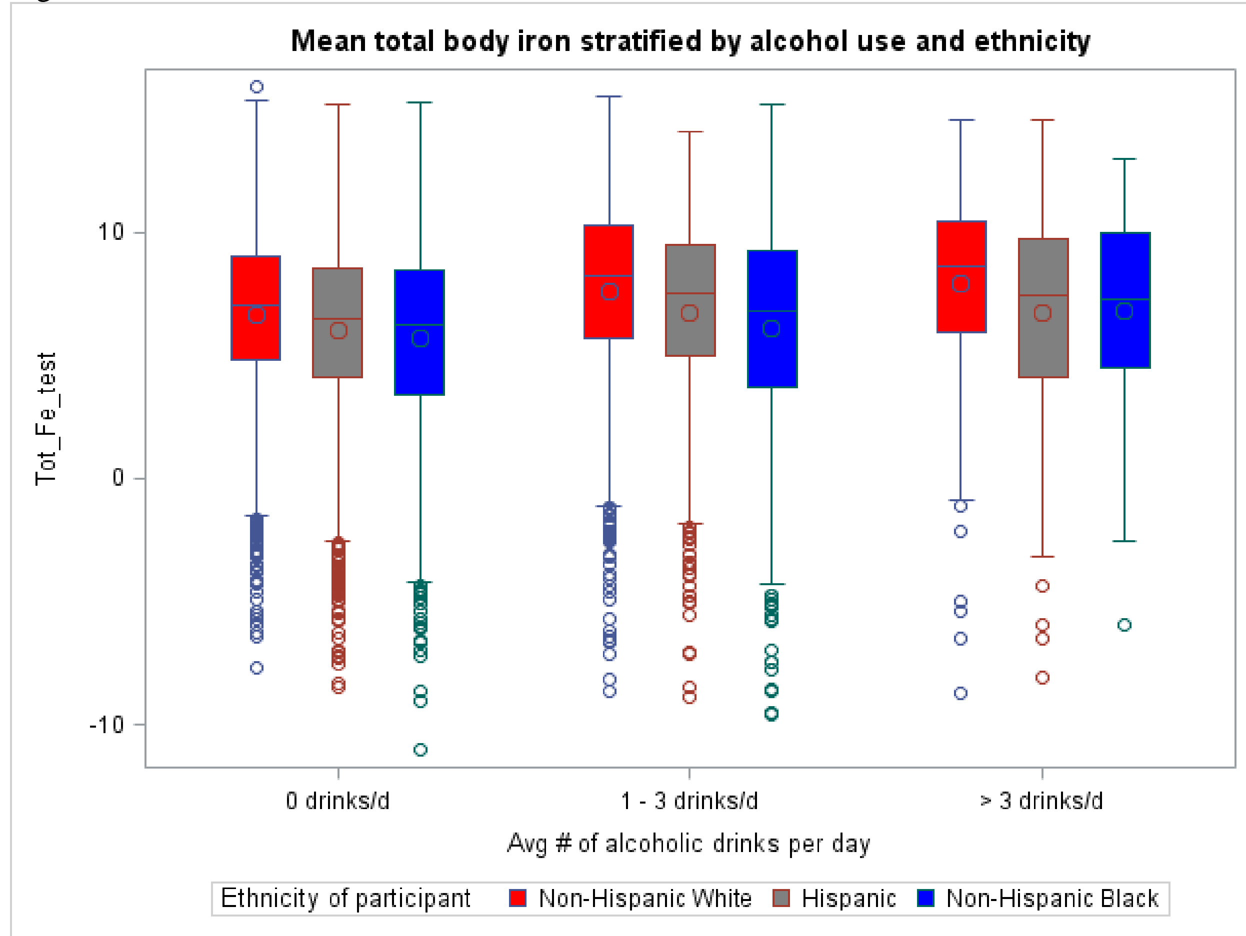

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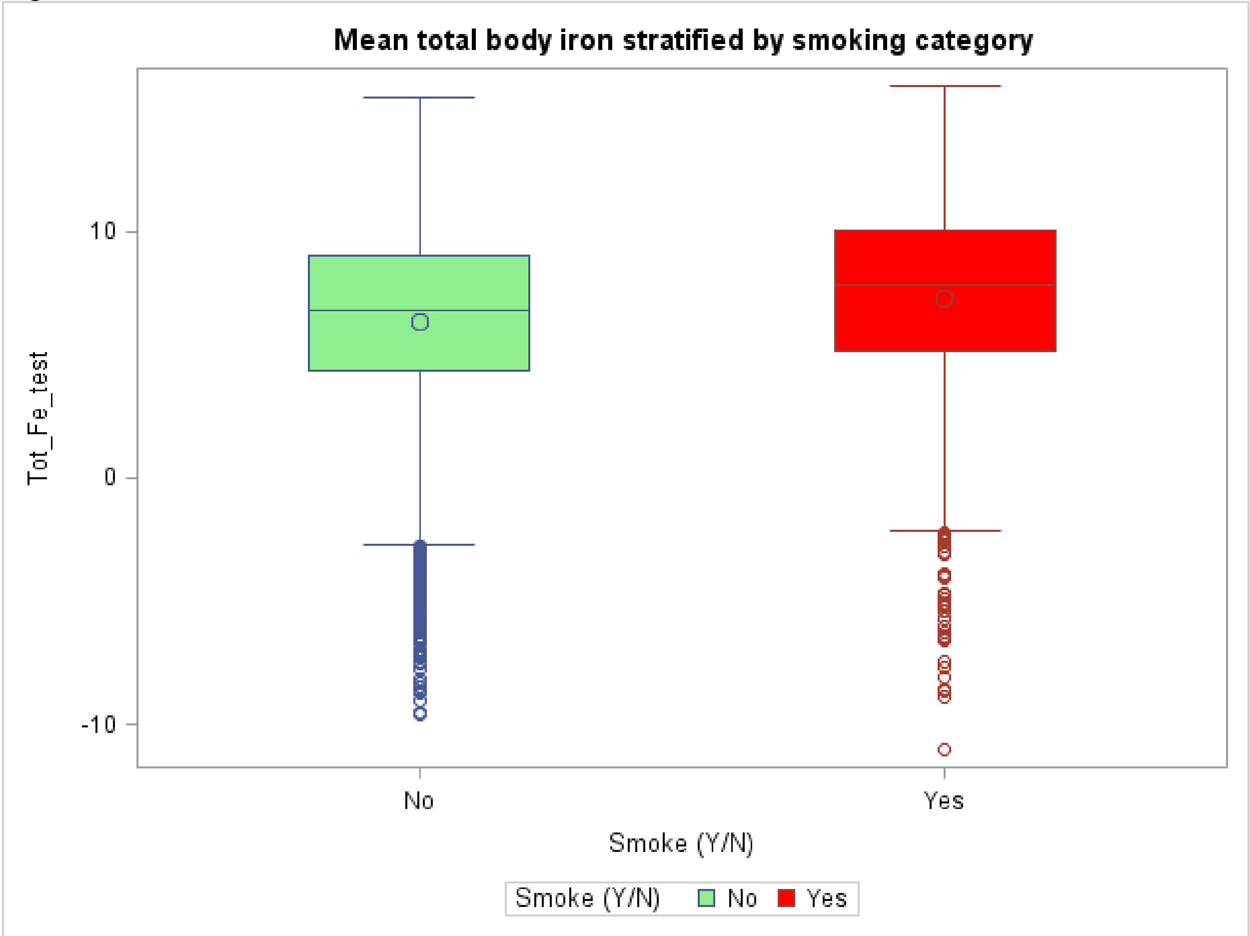
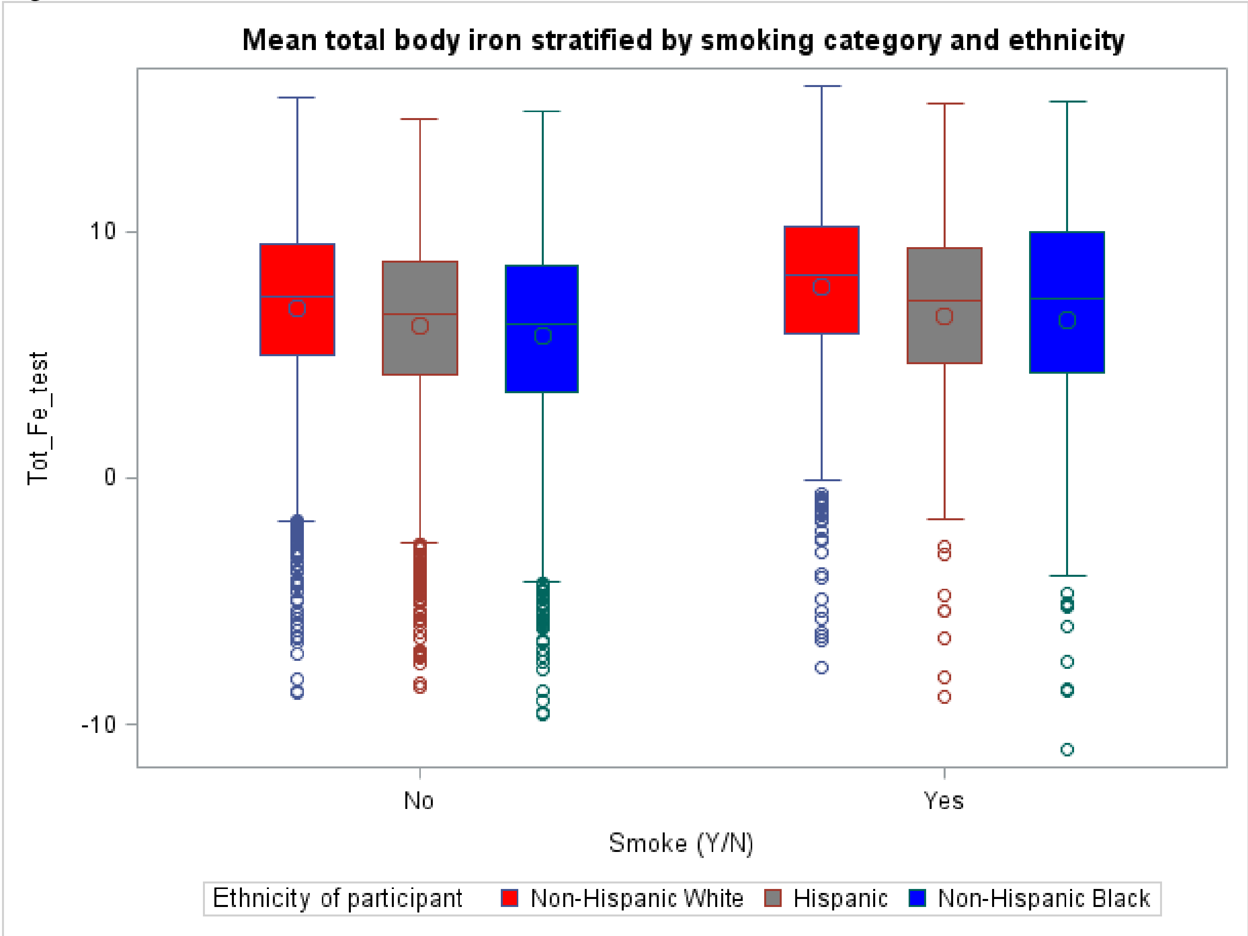


Figure 21



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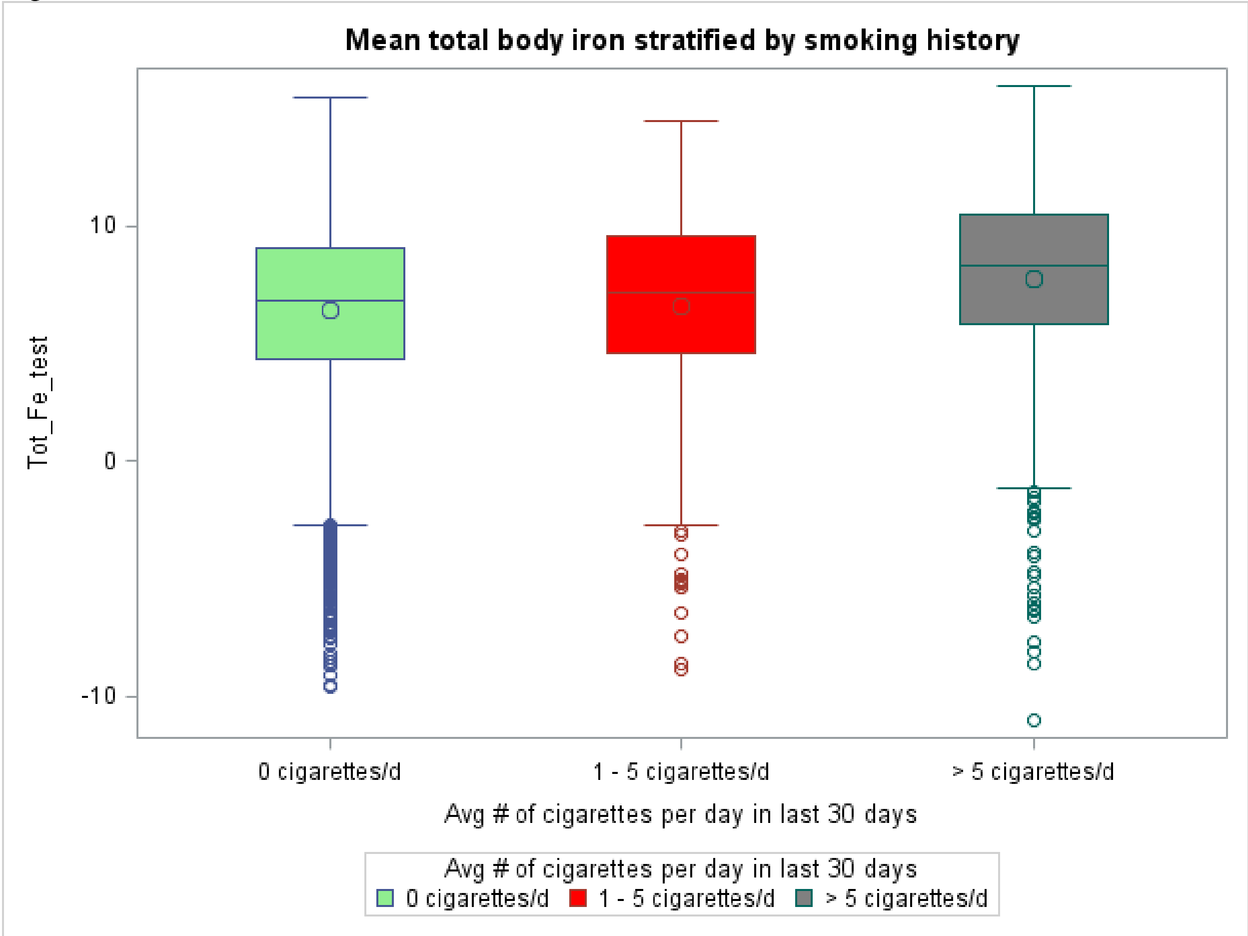

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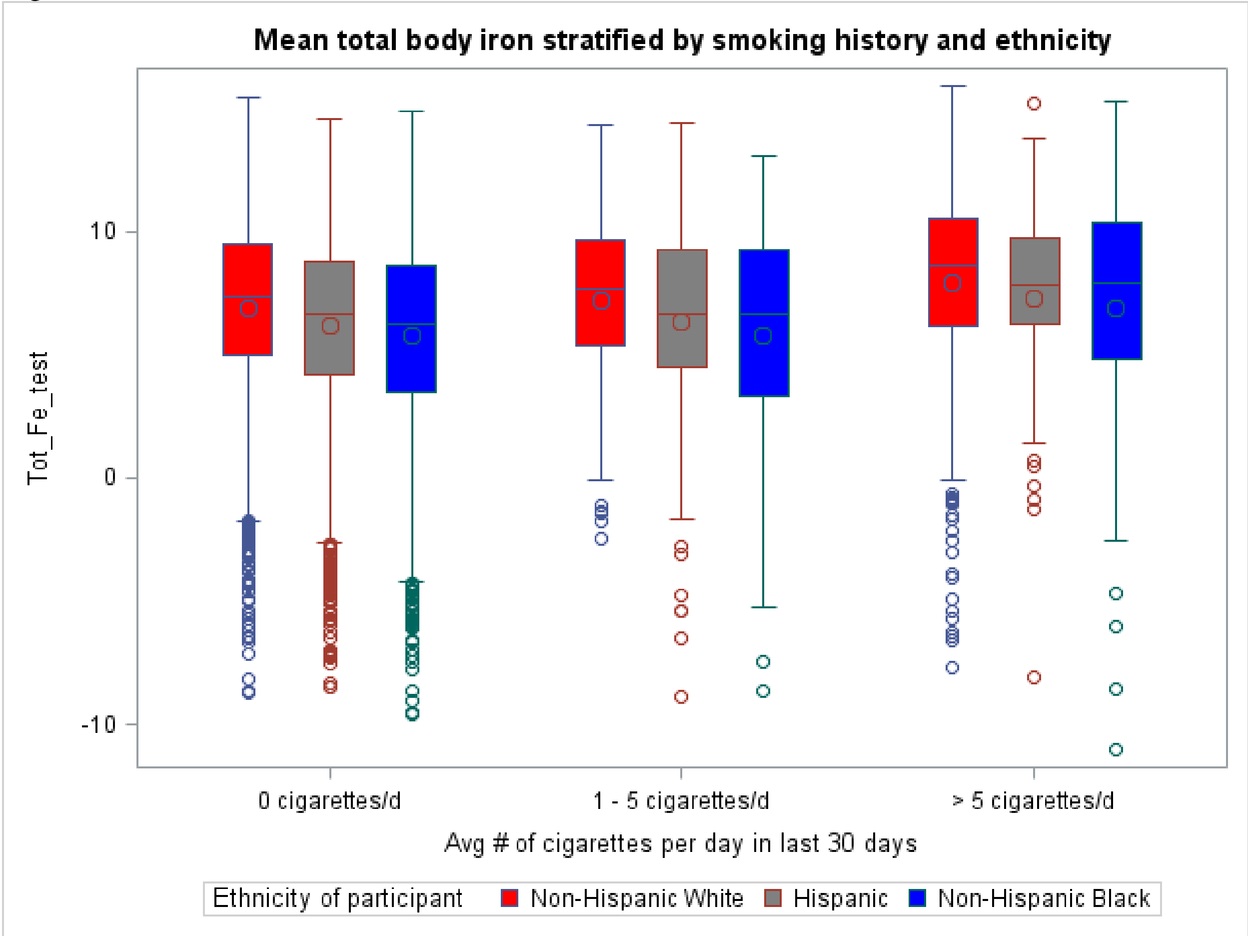
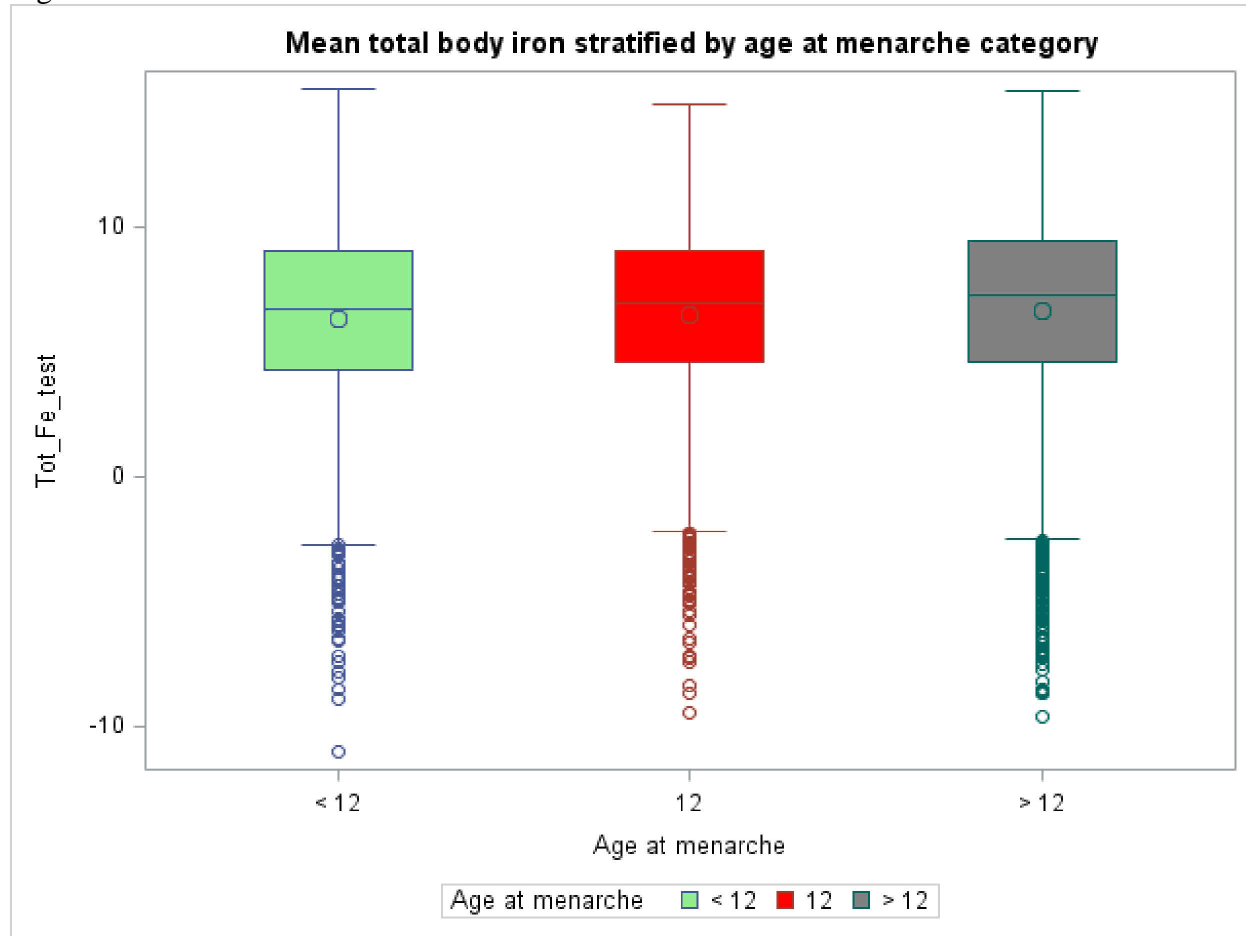


Figure 24



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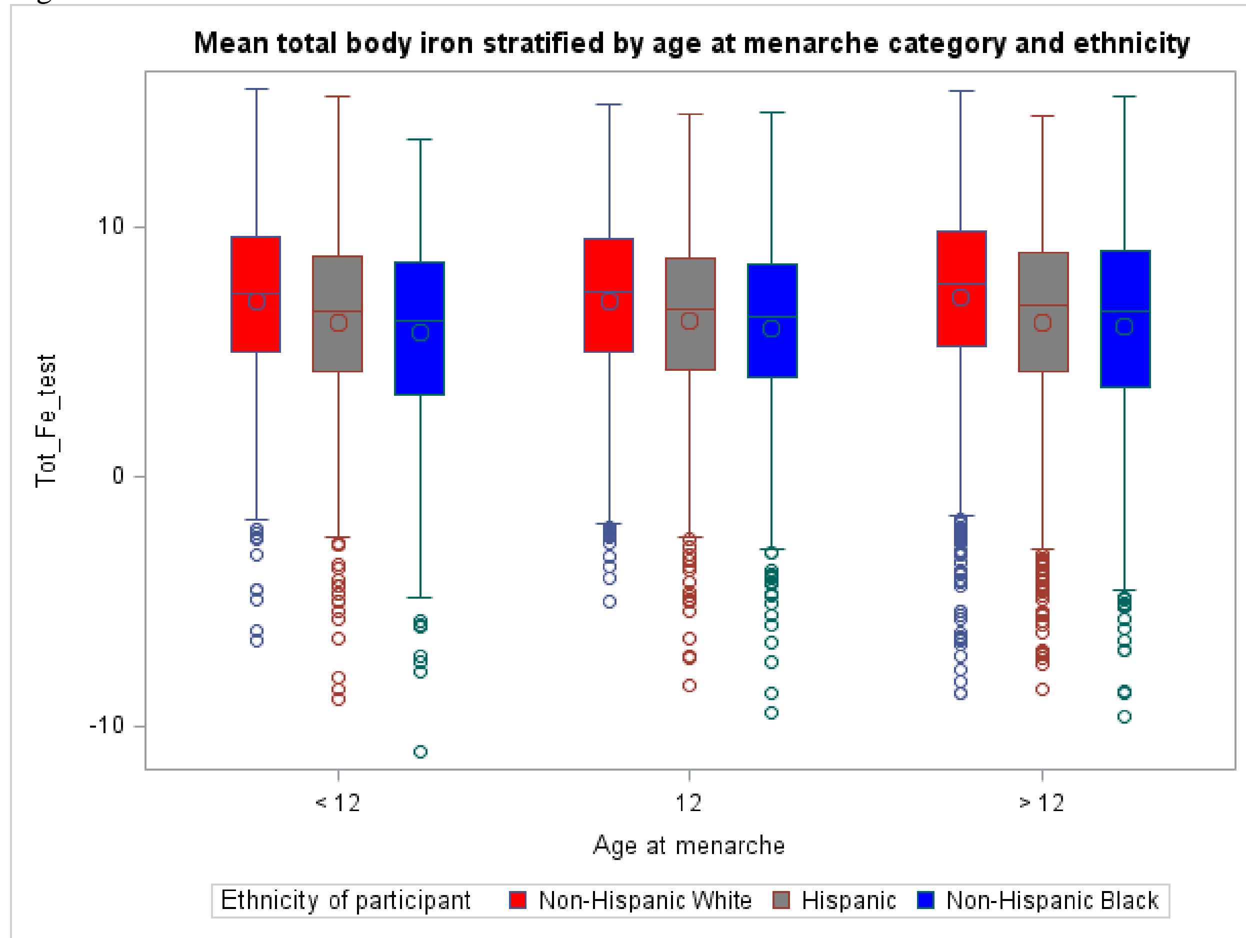

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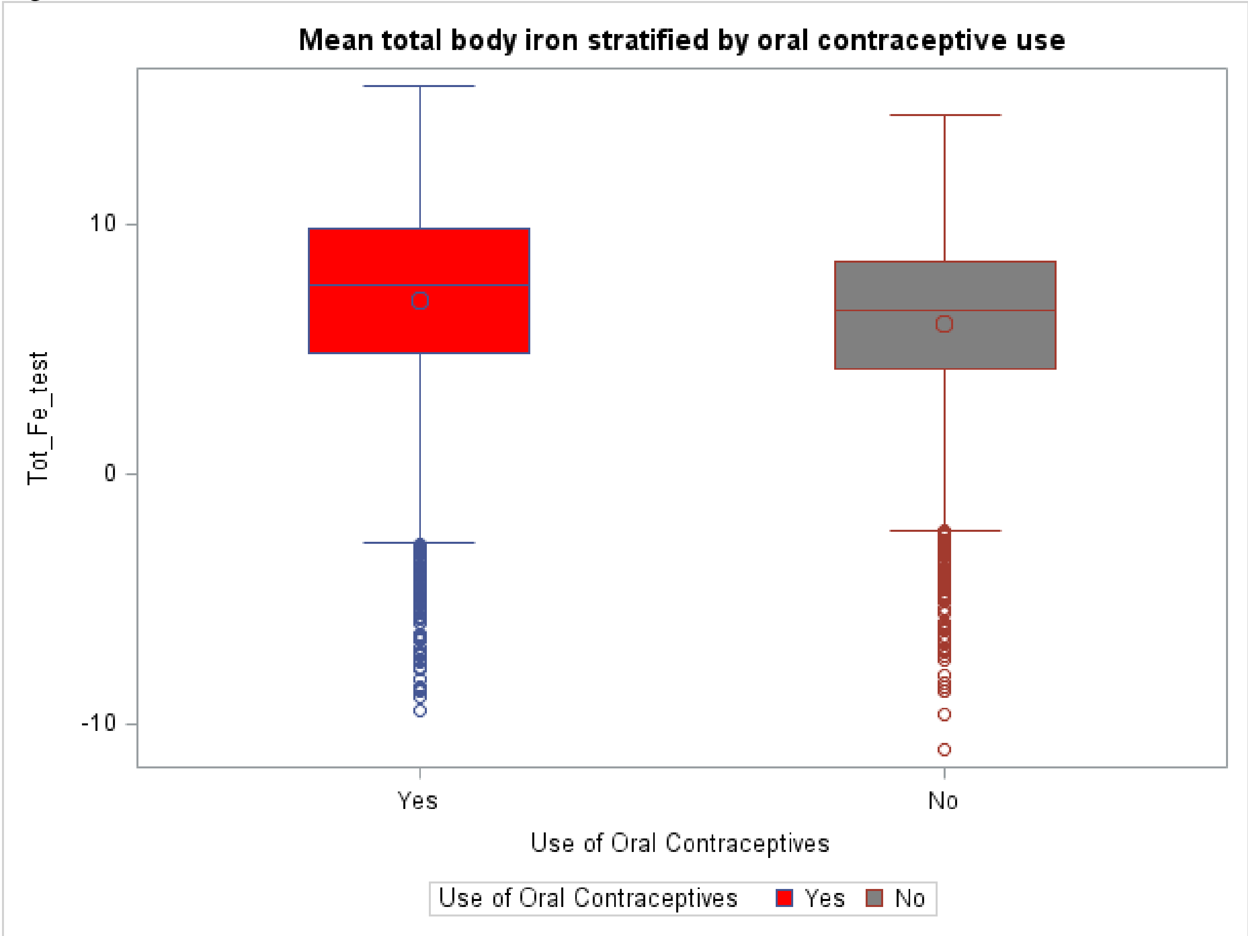

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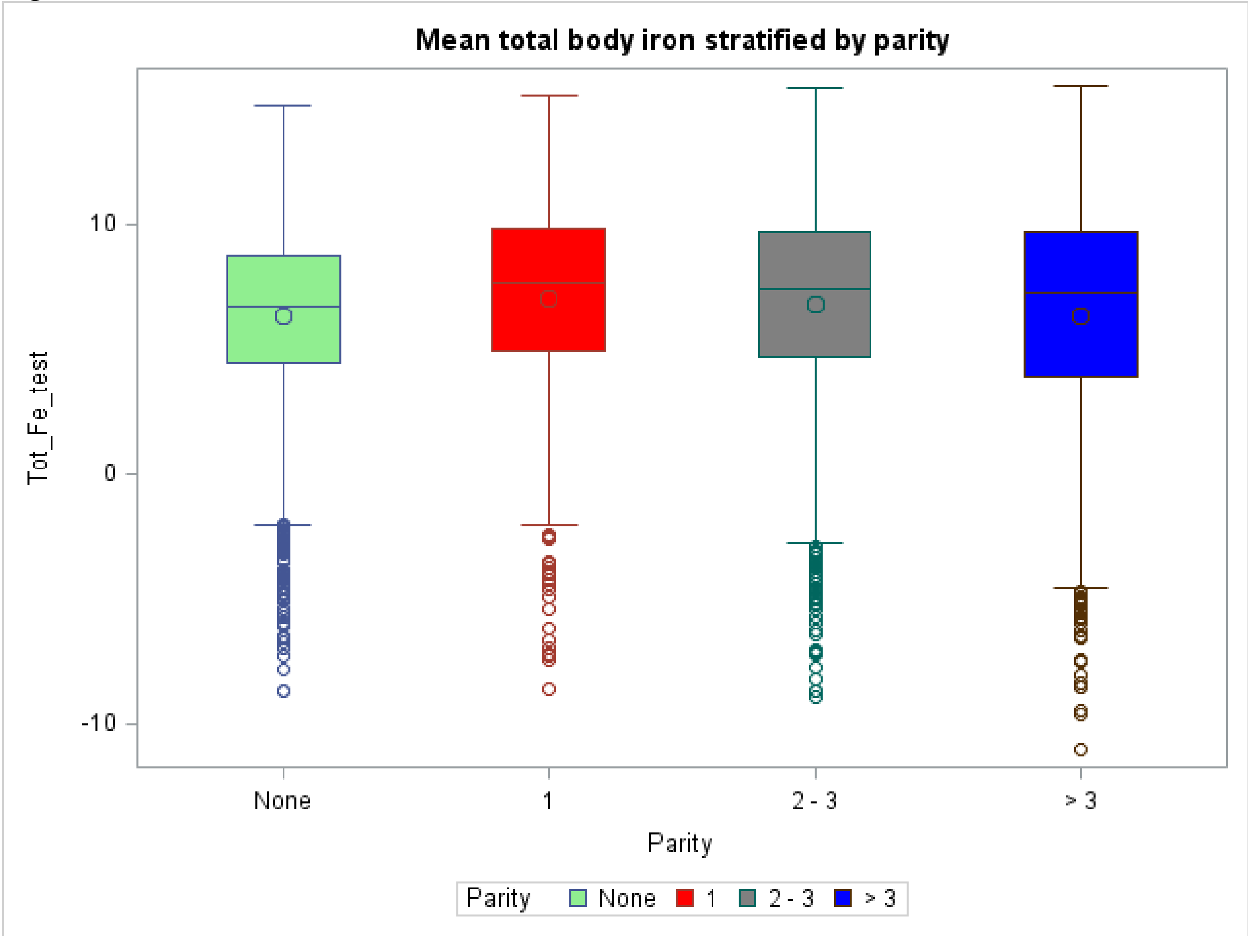

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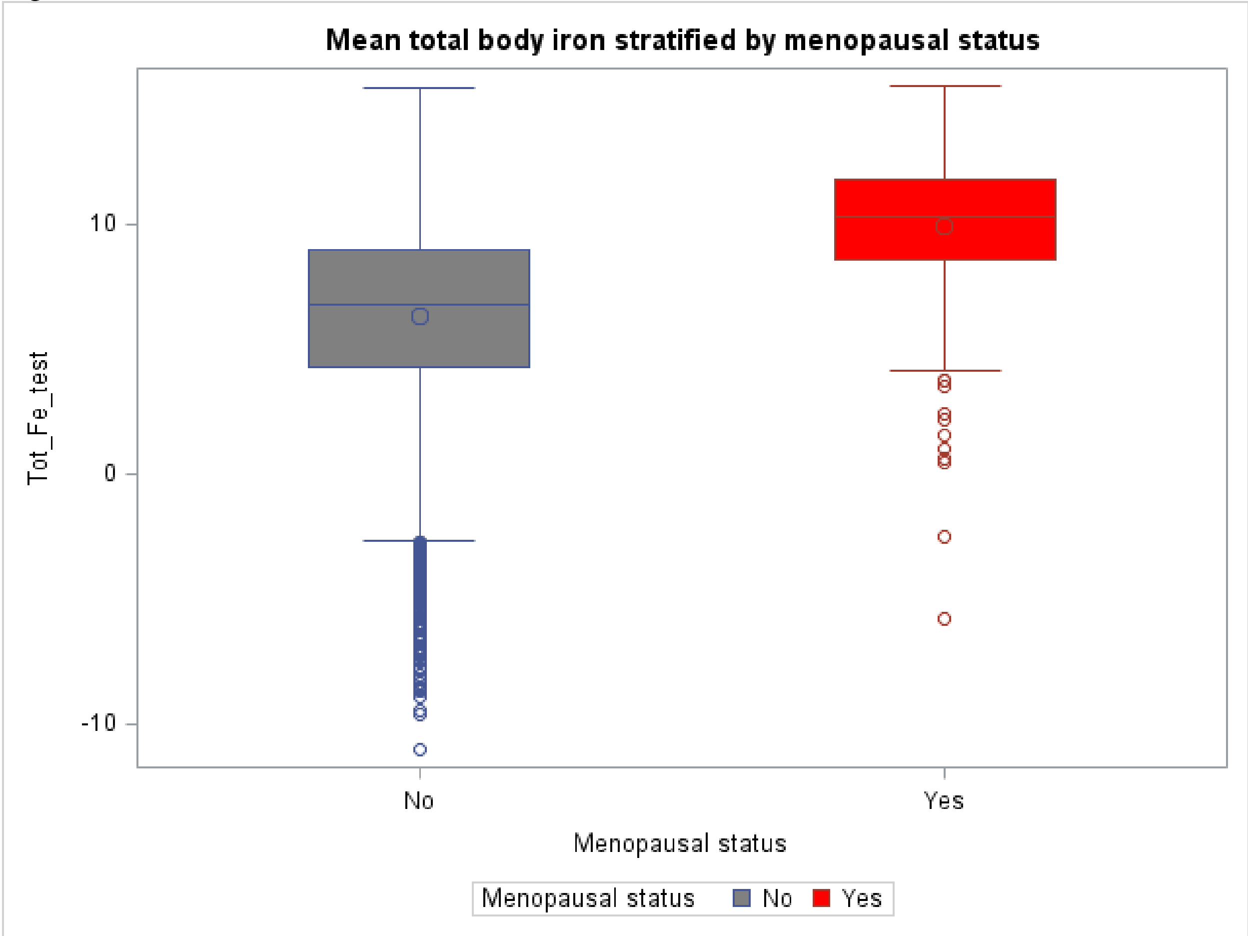

Appendix 1:

Mechanics of Fe_{COOK} So that the reader develops a more complete understanding of the mechanics of Fe_{COOK}, the following section details how the index changes when 1) serum transferrin receptor concentrations increase, holding serum ferritin concentrations constant, 2) serum transferrin receptor decrease, holding serum ferritin concentrations constant, 3) serum ferritin concentrations increase, holding serum transferrin receptor concentrations constant, 4) serum ferritin concentrations decrease, holding serum transferrin receptor concentrations constant, 5) serum transferrin receptor concentrations increase and serum ferritin concentrations decrease, 6) serum transferrin concentrations decrease and serum ferritin concentrations increase, 7) both increase and 8) both decrease. For convenience, the index is given below:

$$Fe_{COOK}\ (mg/kg) = \frac{-\left[\log 10\left(\frac{R*1000}{F}\right)-2.8229\right]}{0.1207},$$

where R = serum transferrin receptor (mg/L) and F = serum ferritin (µg/L). As mentioned, serum transferrin receptor is primarily derived from developing red blood cells, reflects the magnitude of erythropoiesis and the body's demand for iron and is relatively unaffected by inflammation. Ferritin is an intracellular protein whose primary function is iron storage.

As one can see from the simulations below, the index increases substantially when serum transferrin receptor concentrations decrease and serum ferritin concentrations are held constant or increase (simulations 2 and 6, respectively). Simulation 2 represents a scenario where there is a decreased requirement for iron in the presence of stable iron stores. Therefore, if serum ferritin concentrations do not change in the presence of decreasing serum transferrin receptor concentrations, the index

increases. Simulation 6 represents a scenario where there is a decreased requirement for iron coupled with an increase in iron stores, indicative of increased iron absorption with a concomitant increase in Fe_{COOK}. The index increases more slowly when both analytes increase (simulation 7) and this represents a scenario of both an increased requirement for iron with an increase in iron storage. The opposite is true when serum transferrin receptor concentrations increase and serum ferritin concentrations are held constant or decrease (simulations 1 and 5, respectively). Simulation 1 represents a scenario of increasing iron deficiency with stable serum ferritin concentrations. Therefore, if serum ferritin concentrations do not change in the presence of increasing serum transferrin receptor concentrations, the index decreases. Simulation 5 represents a scenario of increasing iron deficiency coupled with decreased iron storage. This scenario might be best visualized within the context of continual blood donation. The index decreases more slowly when both analytes decrease (simulation 8), represented by a scenario where there is a decreased requirement for iron coupled with a decrease in iron stores. When serum transferrin receptor concentrations are held constant, the index increases with increasing serum ferritin concentrations (simulation 3) and decreases with decreasing serum ferritin concentrations (simulation 4). Given that serum transferrin receptor concentrations are only affected by the rate of erythropoiesis and cellular iron content and are unaffected by inflammation, simulation 3 represents a scenario where the behavior of the index can be examined under such conditions (i.e., increases in serum ferritin concentrations due to inflammation). As is evident, the index increases but does so more slowly compared to other simulations.

Simulation	sTfR (mg/L)	ferritin (μg/L)	Fe_{COOK}
1	1	50	12.60870
	1.5	50	11.14978
	2	50	10.11466
	2.5	50	9.31176
	3	50	8.65575
	3.5	50	8.10109
	4	50	7.62063
	4.5	50	7.19683
	5	50	6.81773

Simulation	sTfR (mg/L)	ferritin (μg/L)	Fe_{COOK}
2	5	50	6.81773
	4.5	50	7.19683
	4	50	7.62063
	3.5	50	8.10109
	3	50	8.65575
	2.5	50	9.31176
	2	50	10.11466
	1.5	50	11.14978
	1	50	12.60870

Simulation	sTfR (mg/L)	ferritin (μg/L)	Fe_{COOK}
3	3	50	8.65575
	3	55	8.99869
	3	60	9.31176
	3	65	9.59977
	3	70	9.86642
	3	75	10.11466
	3	80	10.34688
	3	85	10.56502
	3	90	10.77068

Simulation	sTfR (mg/L)	ferritin (μg/L)	Fe_{COOK}
4	3	90	10.77068
	3	85	10.56502
	3	80	10.34688
	3	75	10.11466
	3	70	9.86642
	3	65	9.59977
	3	60	9.31176
	3	55	8.99869
	3	50	8.65575

Simulation	sTfR (mg/L)	ferritin (μg/L)	Fe_{COOK}
5	2.7	90	11.14978
	2.8	85	10.81326
	2.9	80	10.46886
	3.0	75	10.11466
	3.1	70	9.74844
	3.2	65	9.36755
	3.3	60	8.96883
	3.4	55	8.54833
	3.5	50	8.10109

Simulation	sTfR (mg/L)	ferritin (μg/L)	Fe_{COOK}
6	2.5	50	9.31176
	2.4	55	9.80159
	2.3	60	10.26780
	2.2	65	10.71575
	2.1	70	11.14978
	2.0	75	11.57358
	1.9	80	11.99036
	1.8	85	12.40304
	1.7	90	12.81436

Simulation	sTfR (mg/L)	ferritin (μg/L)	Fe_{COOK}
7	2.7	50	9.03485
	2.8	55	9.24693
	2.9	60	9.43375
	3.0	65	9.59977
	3.1	70	9.74844
	3.2	75	9.88245
	3.3	80	10.00394
	3.4	85	10.11466
	3.5	90	10.21603

Simulation	sTfR (mg/L)	ferritin (μg/L)	Fe_{COOK}
8	2.5	90	11.42670
	2.4	85	11.36792
	2.3	80	11.30292
	2.2	75	11.23064
	2.1	70	11.14978
	2.0	65	11.05869
	1.9	60	10.95524
	1.8	55	10.83670
	1.7	50	10.69943

Appendix 2:

Transferrin saturation (TSAT) and Metabolic Dysfunction The odds of T2DM decreased with increasing TSAT (OR = 0.97 95%CI: 0.95-1.00, $P = 0.07$ for each unit increase in TSAT. The effect was slightly amplified after adjusting for covariates. When independently adjusting for 1) age, 2) ethnicity, 3) education, 4) BMI, 5) age at menarche, 6) OC use, 7) parity, 8) menopausal status, 9) alcohol consumption, 10) smoking history, 11) physical activity and 12) C-reactive protein (CRP), the odds of T2DM were 1) 0.97 (95%CI: 0.95-1.00, $P = 0.08$), 2) 0.98 (95%CI: 0.95-1.01, $P = 0.17$), 3) 0.97 (95%CI: 0.95-1.00, $P = 0.08$), 4) 0.97 (95%CI: 0.96-1.03, $P = 0.84$), 5) 0.97 (95%CI: 0.94-1.01, $P = 0.09$), 6) 0.97 (95%CI: 0.94-1.01, $P = 0.09$), 7) 0.97 (95%CI: 0.94-1.01, $P = 0.13$), 8) 0.97 (95%CI: 0.93-1.00, $P = 0.08$), 9) 0.97 (95%CI: 0.95-1.00, $P = 0.09$), 10) 0.97 (95%CI: 0.95-1.00, $P = 0.07$), 11) 0.97 (95%CI: 0.94-1.02, $P = 0.08$) and 12) 0.99 (95%CI: 0.96-1.05, $P = 0.43$) (Table x), respectively. In a full model which adjusted for all covariates, the odds of T2DM did not increase with increasing TSAT (OR = 1.00, 95%CI: 0.96-1.05, $P = 0.91$) (Table 12).

When TSAT was categorized according to tertiles, the odds of T2DM among those in the highest tertile of TSAT compared to those in the lowest tertile were 0.48 (95%CI: 0.26-0.86, $P = 0.01$). A similar pattern was observed after stratifying by ethnicity. Among white females, the odds of T2DM among those in the highest tertile of TSAT compared to those in the lowest tertile were 0.41 (95%CI: 0.17-1.04, $P = 0.06$). The odds of T2DM were higher among Hispanic and black females in the highest tertile of TSAT compared to those in the lowest tertile: OR = 0.70 (95%CI: 0.30-1.66, $P = 0.42$) and OR = 0.74 (95%CI: 0.23-2.40, $P = 0.61$).

The odds of metabolic syndrome decreased with increasing TSAT (OR = 0.97 95%CI: 0.95-0.98, $P < 0.0001$ for each unit increase in TSAT. The effect was slightly amplified after adjusting for individual covariates in the full model. When independently adjusting for 1) age, 2) ethnicity, 3) education, 4) BMI, 5) age at menarche, 6) OC use, 7) parity, 8) menopausal status, 9) alcohol consumption, 10) smoking history, 11) physical activity and 12) C-reactive protein (CRP), the odds of metabolic syndrome were 1) 0.96 (95%CI: 0.95-0.98, $P < 0.0001$), 2) 0.96 (95%CI: 0.95-0.98, $P < 0.0001$), 3) 0.96 (95%CI: 0.95-0.98, $P < 0.0001$), 4) 0.99 (95%CI: 0.97-1.01, $P = 0.21$), 5) 0.96 (95%CI: 0.95-0.98, $P < 0.0001$), 6) 0.96 (95%CI: 0.95-0.98, $P < 0.0001$), 7) 0.97 (95%CI: 0.95-0.98, $P < 0.0001$), 8) 0.96 (95%CI: 0.94-0.98, $P < 0.0001$), 9) 0.96 (95%CI: 0.95-0.98, $P < 0.0001$), 10) 0.96 (95%CI: 0.95-0.98, $P < 0.0001$), 11) 0.96 (95%CI: 0.95-0.98, $P < 0.0001$) and 12) 0.97 (95%CI: 0.98-0.99, $P = 0.0003$) (Table 12), respectively. In a full model which adjusted for all covariates, the odds of metabolic syndrome slightly increased to 0.98 (95%CI: 0.96-1.01), which was no longer statistically significant ($P = 0.14$) (Table 12).

When TSAT was categorized according to tertiles, the odds of metabolic syndrome among those in the highest tertile of TSAT compared to those in the lowest tertile were 0.46 (95%CI: 0.30-0.70, $P = 0.0003$). A similar pattern was observed after stratifying by ethnicity. Among white females, the odds of metabolic syndrome among those in the highest tertile of TSAT compared to those in the lowest tertile were 0.43 (95%CI: 0.25-0.76, $P = 0.004$). The odds of metabolic syndrome were higher among Hispanic and black females in the highest tertile of TSAT compared to those in the lowest

tertile: OR = 0.53 (95%CI: 0.25-1.13, $P = 0.10$) and OR = 0.49 (95%CI: 0.22-1.09, $P = 0.08$), respectively.

TSAT was inversely associated with U.S.-FLI. For each unit increase in U.S.-FLI, TSAT decreased 0.15% ($P < 0.0001$). Similarly, for each unit increase in TSAT, U.S.-FLI decreased 0.31 units ($P < 0.0001$). The odds of NAFLD decreased with increasing TSAT (OR = 0.96, 95%CI: 0.94-0.97, $P < 0.0001$ for each unit increase in TSAT. The effect was slightly amplified in the full model after adjusting for covariates. When independently adjusting for 1) age, 2) ethnicity, 3) education, 4) BMI, 5) age at menarche, 6) OC use, 7) parity, 8) menopausal status, 9) alcohol consumption, 10) smoking history, 11) physical activity and 12) C-reactive protein (CRP), the odds of NAFLD were 1) 0.96 (95%CI: 0.94-0.98, $P < 0.0001$), 2) 0.96 (95%CI: 0.94-0.98, $P < 0.0001$), 3) 0.96 (95%CI: 0.94-0.98, $P < 0.0001$), 4) 0.98 (95%CI: 0.96-1.01, $P = 0.13$), 5) 0.96 (95%CI: 0.94-0.98, $P < 0.0001$), 6) 0.96 (95%CI: 0.94-0.98, $P < 0.0001$), 7) 0.96 (95%CI: 0.94-0.98, $P < 0.0001$), 8) 0.96 (95%CI: 0.94-0.98, $P < 0.0001$), 9) 0.95 (95%CI: 0.93-0.97, $P < 0.0001$), 10) 0.95 (95%CI: 0.93-0.97, $P < 0.0001$), 11) 0.95 (95%CI: 0.93-0.97, $P < 0.0001$) and 12) 0.97 (95%CI: 0.96-0.99, $P = 0.001$) (Table x), respectively. In a full model which adjusted for all covariates, no increased odds of NAFLD as a function of TSAT were observed (OR = 0.99, 95%CI: 0.96-1.01, $P = 0.26$) (Table 12).

When TSAT was categorized according to tertiles, the odds of NAFLD decreased across increasing tertiles ($P_{trend} < 0.0001$). Compared to those in the lowest tertile of TSAT the odds of NAFLD were 0.62 (95%CI: 0.39-0.97, $P = 0.04$) for those in the middle tertile of TSAT. Compared to those in the lowest tertile of TSAT, the odds of NAFLD were 0.41 (95%CI: 0.28-0.61, $P < 0.0001$) among those in the highest tertile. A

similar pattern was observed after stratifying by ethnicity. Among white females, the odds of NAFLD among those in the highest tertile of TSAT compared to those in the lowest tertile were 0.39 (95%CI: 0.22-0.69, $P = 0.001$). The odds of NAFLD were higher among Hispanic females in the highest tertile of TSAT compared to those in the lowest tertile: OR = 0.40 (95%CI: 0.17-0.90, $P = 0.02$). Compared to their white and Hispanic counterparts, the odds of NAFLD were higher among black females in the highest tertile of TSAT compared to those in the lowest tertile: OR = 0.69 (95%CI: 0.29-1.64, $P = 0.40$). TSAT was also inversely associated with liver fibrosis (i.e., NFS). For each unit increase in NFS, TSAT decreased 0.56% ($P = 0.01$). Similarly, for each unit increase in TSAT, NFS decreased 0.01 units ($P = 0.02$).

TSAT was inversely associated with insulin resistance. For each unit increase in HOMA-IR, TSAT decreased 0.60% ($P = 0.002$). Similarly, for each unit increase in TSAT, HOMA-IR decreased 0.04 units ($P < 0.0001$). The odds of insulin resistance decreased with increasing TSAT (OR = 0.97, 95%CI: 0.95-0.99, $P = 0.01$) for each unit increase in TSAT. The effect was slightly amplified after adjusting for covariates. When independently adjusting for 1) age, 2) ethnicity, 3) education, 4) BMI, 5) age at menarche, 6) OC use, 7) parity, 8) menopausal status, 9) alcohol consumption, 10) smoking history, 11) physical activity and 12) C-reactive protein (CRP), the odds of insulin resistance were 1) 0.97 (95%CI: 0.95-0.99, $P = 0.01$), 2) 0.98 (95%CI: 0.97-0.99, $P = 0.03$), 3) 0.98 (95%CI: 0.96-1.00, $P = 0.06$), 4) 0.98 (95%CI: 0.95-0.99, $P = 0.02$), 5) 0.99 (95%CI: 0.96-1.02, $P = 0.51$), 6) 0.98 (95%CI: 0.95-1.00, $P = 0.06$), 7) 0.97 (95%CI: 0.95-1.00, $P = 0.05$), 8) 0.97 (95%CI: 0.94-0.99, $P = 0.03$), 9) 0.97 (95%CI: 0.95-0.99, $P = 0.04$), 10) 0.97 (95%CI: 0.94-0.99, $P = 0.01$), 11) 0.96 (95%CI: 0.94-

0.99, $P = 0.002$) and 12) 0.98 (95%CI: 0.96-1.01, $P = 0.16$) (Table x), respectively. In a full model which adjusted for all covariates, the odds of insulin resistance did not increase with increasing TSAT (OR = 1.00 95%CI: 0.97-1.04, $P = 0.93$) (Table 12).

When TSAT was categorized according to tertiles, the odds of insulin resistance decreased across increasing tertiles ($P_{trend} = 0.06$). Compared to those in the lowest tertile of TSAT the odds of insulin resistance were 0.75 (95%CI: 0.44-1.28, $P = 0.29$) for those in the middle tertile of TSAT. Compared to those in the lowest tertile of TSAT, the odds of insulin resistance were 0.50 (95%CI: 0.28-0.90, $P = 0.02$) among those in the highest tertile of TSAT. A similar pattern was observed after stratifying by ethnicity, especially among white females. Among white females, the odds of insulin resistance among those in the highest tertile of TSAT compared to those in the lowest tertile were 0.56 (95%CI: 0.23-1.40, $P = 0.28$). The odds of insulin resistance were also higher among Hispanic and black females in the highest tertile of TSAT compared to those in the lowest tertile: OR = 0.48 (95%CI: 0.17-1.34, $P = 0.19$) and OR = 0.74 (95%CI: 0.30-1.83, $P = 0.77$), respectively.

Appendix 3: Power Calculations

Insulin Resistance:

```
proc power;
	twosamplefreq test=pchi
		relativerisk = 1.2 1.3 1.4 1.5
			refproportion = 0.35
			ntotal = 3149
			sides = 2
			alpha = 0.0125
			power = . ;
run;
```

Non-alcoholic fatty liver disease:

```
proc power;
	twosamplefreq test=pchi
		relativerisk = 1.2 1.3 1.4 1.5
			refproportion = 0.291
			ntotal = 3057
			sides = 2
			alpha = 0.0125
			power = . ;
run;
```

Metabolic Syndrome:

```
proc power;
	twosamplefreq test=pchi
		relativerisk = 1.2 1.3 1.4 1.5
			refproportion = 0.234
			ntotal = 6511
			sides = 2
			alpha = 0.0125
			power = . ;
run;
```

Type 2 diabetes mellitus:

```
proc power;
	twosamplefreq test=pchi
		relativerisk = 1.2 1.3 1.4 1.5
			refproportion = 0.11
			ntotal = 6841
			sides = 2
			alpha = 0.0125
			power = . ;
run;
```

Table 16: Power and sample size calculations: T2DM					
Outcome of interest	Population prevalence (i.e., reference proportion) in females	Year of NHANES survey	Total sample size*	Relative Risk	Computed Power**
Type 2 diabetes mellitus	11.2%	2003-2004	2468	1.2	0.40
	11.2%		2468	1.3	0.70
	11.2%		2468	1.4	0.90
	11.2%		2468	1.5	0.98
	11.2%	2005-2006	2682	1.2	0.42
	11.2%		2682	1.3	0.74
	11.2%		2682	1.4	0.93
	11.2%		2682	1.5	0.99
	11.2%	2007-2008	2109	1.2	0.35
	11.2%		2109	1.3	0.63
	11.2%		2109	1.4	0.86
	11.2%		2109	1.5	0.96
	11.2%	2009-2010	2512	1.2	0.40
	11.2%		2512	1.3	0.71
	11.2%		2512	1.4	0.91
	11.2%		2512	1.5	0.98
	11.2%	2003-2010 (pooled)	9771	1.2	0.92
	11.2%		9771	1.3	0.99
	11.2%		9771	1.4	> 0.99
	11.2%		9771	1.5	> 0.99

**Total sample size refers to the number of females during each year who have complete data for the calculation for* Tot_{Fe} *and Non-alcoholic fatty liver disease.*

***See appendix for SAS code used to calculate power.*

Table 17: Power and sample size calculations: Metabolic syndrome

Outcome of interest	Outcome of interest	Outcome of interest	Outcome of interest	Outcome of interest	Outcome of interest
Metabolic syndrome					
	11.2%		2468	1.3	0.70
	11.2%		2468	1.4	0.90
	11.2%		2468	1.5	0.98
	11.2%	2005-2006	2682	1.2	0.42
	11.2%		2682	1.3	0.74
	11.2%		2682	1.4	0.93
	11.2%		2682	1.5	0.99
	11.2%	2007-2008	2109	1.2	0.35
	11.2%		2109	1.3	0.63
	11.2%		2109	1.4	0.86
	11.2%		2109	1.5	0.96
	11.2%	2009-2010	2512	1.2	0.40
	11.2%		2512	1.3	0.71
	11.2%		2512	1.4	0.91
	11.2%		2512	1.5	0.98
	11.2%	2003-2010 (pooled)	9771	1.2	0.92
	11.2%		9771	1.3	0.99
	11.2%		9771	1.4	> 0.99
	11.2%		9771	1.5	> 0.99

**Total sample size refers to the number of females during each year who have complete data for the calculation for Tot_{Fe} and Non-alcoholic fatty liver disease.*

***See appendix for SAS code used to calculate power.*

Table 18: Power and sample size calculations: Non-alcoholic fatty liver disease					
Outcome of interest	Outcome of interest	Outcome of interest	Outcome of interest	Outcome of interest	Outcome of interest
Non-alcoholic fatty liver disease (y = U.S.-FLI)					
	11.2%		2468	1.3	0.70
	11.2%		2468	1.4	0.90
	11.2%		2468	1.5	0.98
	11.2%	2005-2006	2682	1.2	0.42
	11.2%		2682	1.3	0.74
	11.2%		2682	1.4	0.93
	11.2%		2682	1.5	0.99
	11.2%	2007-2008	2109	1.2	0.35
	11.2%		2109	1.3	0.63
	11.2%		2109	1.4	0.86
	11.2%		2109	1.5	0.96
	11.2%	2009-2010	2512	1.2	0.40
	11.2%		2512	1.3	0.71
	11.2%		2512	1.4	0.91
	11.2%		2512	1.5	0.98
	11.2%	2003-2010	9771	1.2	0.92
	11.2%	(pooled)	9771	1.3	0.99
	11.2%		9771	1.4	> 0.99
	11.2%		9771	1.5	> 0.99

**Total sample size refers to the number of females during each year who have complete data for the calculation for Tot_{Fe} and Non-alcoholic fatty liver disease.*

***See appendix for SAS code used to calculate power.*

Table 19: Power and sample size calculations: insulin resistance

Outcome of interest	Outcome of interest	Outcome of interest	Outcome of interest	Outcome of interest	Outcome of interest
Insulin resistance (y = HOMA-IR)					
	11.2%		2468	1.3	0.70
	11.2%		2468	1.4	0.90
	11.2%		2468	1.5	0.98
	11.2%	2005-2006	2682	1.2	0.42
	11.2%		2682	1.3	0.74
	11.2%		2682	1.4	0.93
	11.2%		2682	1.5	0.99
	11.2%	2007-2008	2109	1.2	0.35
	11.2%		2109	1.3	0.63
	11.2%		2109	1.4	0.86
	11.2%		2109	1.5	0.96
	11.2%	2009-2010	2512	1.2	0.40
	11.2%		2512	1.3	0.71
	11.2%		2512	1.4	0.91
	11.2%		2512	1.5	0.98
	11.2%	2003-2010 (pooled)	9771	1.2	0.92
	11.2%		9771	1.3	0.99
	11.2%		9771	1.4	> 0.99
	11.2%		9771	1.5	> 0.99

**Total sample size refers to the number of females during each year who have complete data for the calculation for* Tot_{Fe} *and Non-alcoholic fatty liver disease.*

***See appendix for SAS code used to calculate power.*

CURRICULUM VITAE

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Education

2017	Ph.D.	Epidemiology *Specialization: Genetic Epidemiology* *Biostatistics minor*	University of Louisville
2010	M.A.	Educational & Developmental Psychology *Physiological Psychology minor*	University of Louisville
2000	B.S.	Computer Science	Morehead State University

Summary

My skill set is most effectively applied to solving problems with research, mathematics, epidemiology, biostatistics, data management (e.g., data warehousing, relational databases, data cleaning), data analysis (e.g., parametric modeling, nonparametric modeling, performance metrics, business intelligence), simulations (e.g., algorithm optimization, risk analysis, sensitivity analysis, predictive modeling).

I implement the aforementioned skill set using SAS, R, PL/SQL Developer, SQL Server, MySQL, Python, QlikView, Perl, JMP, Access and Excel (VBA).

Research and Clinical Interests

Psychiatric Pharmacogenomics, Cancer Epidemiology, Genetic Epidemiology, Aging Immunology, Tumor Immunology, Childhood Cancer, Maternal & Child Health.

Professional Employment Experience

2016-present Analytics Consultant Humana, Inc.

Work with team to maintain current version of a dynamic claims-based provider attribution algorithm which incorporates member and provider attributes such as geographical distance, provider specialty and a variety of proprietary business rules in order to establish value-based member-provider (PCP) assignments.

Developed and implemented a method to examine the financial impact (i.e., claims spend, premiums spend, MER) of a provider attribution algorithm among members whose PCP was determined by self-selection, Humana assignment (i.e., proprietary business rules), claims-based assignment.

Worked with team to complete a Six-Sigma Green Belt project which examined root causes of inaccurate member-provider assignments. Deliverable to leadership included recommending people, processes and technology to improve member/provider experience with business impacts being accurate panel lists and related STARS quality data & rewards and improved relationships/trust with participating PCPs.

Developed and maintain a fuzzy matching algorithm using SAS functions SPEDIS, SOUNDEX, CONTAINS, COMPARE, COMPGED, PRXPARSE, PRXMATCH to evaluate the concordance between member-PCP relationships across two databases. Automated said project with Microsoft Access.

Developing a prescriber attribution model which quantifies the effect of incorporating pharmacy claims into provider attribution models which do not utilize said data. Developed conditional logic which allows members to be attributed to a prescribing provider when no other claims (e.g., AWV, E&M) are available.

Developed a novel metric for use in the calculation of continuity of care among member-provider networks. The equation takes into account within-provider prescribing behavior that can lead to drug-drug interactions as well as between-provider prescribing behavior that can lead to additional drug-drug interactions.

Developed an annotated training manual for future Attribution Analytics associates.

Developing a PCP Treating model using graph theory and social network analysis and an advanced personalized member model.

2012–2016 Biostatistician Assurex Health, Inc.

Using a priori biological knowledge and preselected SNPs within a finite set of xenobiotic metabolizing and transport genes, assist in the development of clinical decision support algorithms which classify and predict pharmacokinetic and pharmacodynamic response to a panel of commonly prescribed psychotropic and analgesic medications by reducing the dimensionality inherent to permutations and combinations of drug-drug, drug-gene, and gene-gene interactions.

Conduct cross-validation analyses of results obtained from previous in-house clinical trials. Head of using a systematic and methodological approach to data imputation in order to account for the differential effect of subject (i.e., treated vs control) attrition in the above mentioned clinical trials.

Following testing for clinical, methodological, and statistical heterogeneity, merge data from in-house clinical trials of similar design to derive pooled effect sizes, which in turn are used to inform sample size calculation for future trials.

Conducted a current (as of Dec 2012) meta-analysis of the published literature reporting upon the effect of the -759C/T HTR2C transition on antipsychotic-induced weight-gain (AIWG), stratified by drug-experienced versus drug-naive subjects. Random-effects models were employed to account for heterogeneity among study designs. Publication bias was examined via funnel plots and Egger's regression.

Lead biostatistician of a recently launched large-scale (projected N = 1350), treatment-resistant depression (TRD), randomized, controlled trial examining the clinical utility and clinical validity of a multigenic combinatorial phenotype method for selecting genetically-concordant antidepressant medications.

Executed sensitivity analyses for TRD RCT solving for sample size, alpha, and power in order to provide study sponsor with information to guide strategic decision making with respect to overall study recruitment.

Using the CRF implemented in the above TRD RCT as guidance, created a data schema to standardize and accommodate all previous/ongoing clinical trial data. Developed a clinical database/data warehouse thereafter using MySQL to store standardized data.

Co-developed a novel data reduction method which facilitates pairwise comparisons across single enzyme phenotypes and a composite (5 enzyme) phenotype.

Head of creation and management of clinical trials dossier.

Co-developed a methodology to evaluate the performance of several versions of the same clinical decision support algorithm via simulation. Head of automation and statistical analyses of simulations using SAS & R.

Co-developed the methodology to calculate company-wide sales metrics based upon physician ordering patterns. Independently created a SAS program to automate monthly and quarterly calculations.

Co-developed the methodology to calculate 13 KPIs for the Medical Affairs/Clinical Development department within the company. Independently created a SAS program to automate monthly and quarterly calculations.

2010-present Assistant Professor Jefferson Community & Technical College

Full-time professor of three psychology courses at the Carrollton, KY extension campus.

Courses taught: PSY110 (General Psychology), PSY223 (Developmental Psychology), PSY230 (Psychological Aspects of Aging, Dying, Death, & Bereavement.

Direct undergraduate students in the stepwise process of curating academic literature from various databases in order to construct annotated bibliographies, which in turn are employed in the development of a term paper of publishable quality; assist students in the interpretation of academic literature; develop and deploy psychology courses on Blackboard; deliver fifteen lectures per course per semester.

Research Experience

2014**:** Project Manager, School of Public Health and Information Sciences, Department of Epidemiology and Population Health, University of Louisville.
Supervisor: Frank D. Groves, M.D., M.P.H.

Conducted a secondary analysis of a multistage, nationally-representative probabilistic sample of NHANES I/NHEFS linked databases examining the role of hematological parameters on subsequent development of cardiovascular disease and cancer. Kaplan-Meier curves were constructed to estimate differential survival among sample population, stratified by each hematological parameter. Cox proportional hazards regression analyses were employed derive hazard ratios associated with each parameter, stratified by gender and ethnicity.

2013: Project Manager, School of Public Health and Information Sciences, Department of Epidemiology and Population Health, University of Louisville.
Supervisor: Kira Taylor, Ph.D., M.S.

Completed and submitted manuscript proposal for a candidate gene study examining genetic variation in the PI3k/Akt/mTOR pathway and subsequent development of pathological left ventricular hypertrophy in Jackson Heart Study population. Bayesian network analysis (BNA) was employed using R software to curate a concise set of genes within the signaling pathway. Validity and robustness of BNA was ascertained using likelihood ratio tests and bootstrapping for cross-validation. Multi-factor dimensionality reduction (MDR) was employed to finalize a set of tagging SNPs from

selected genes for statistical modeling to reduce the effect of multiple testing. Principal components analysis was employed to reduce multicollinearity among covariates in final logistic regression model.

2013: Project Manager, School of Public Health and Information Sciences, Department of Epidemiology and Population Health, University of Louisville.
Supervisor: Elizabeth O'Brien, Ph.D.

Conducted an exploratory simulation to model the dependency of genetic expression on genetic variants within PI3K/Akt/mTOR signaling pathway to augment the above candidate gene study. Using a priori biological knowledge, dynamic Bayesian network analysis was employed using R and Markov chain Monte Carlo and Python software to account for time-varying probabilistic modeling of RNA expression. Normalized mutual information (MI) between genetic variants and gene expression was calculated to derive statistical models with greatest maximum likelihood. Chi-square tests were then applied to test statistical significance of the final set of MIs.

2012: Project Collaborator, School of Public Health and Information Sciences, Department of Epidemiology and Population Health, University of Louisville.
Supervisor: Rudolph Parrish, Ph.D.

Collaborated on secondary data analysis to explore age-period and site-related effects of air pollution and passive smoking on the development of wheezing in two cohorts of children. T-tests were employed to quantify differential demographic characteristics with continuous outcomes and chi-squared tests were employed to examine differential demographic characteristics with categorical outcomes. Generalized estimating equations were employed to model categorical time-dependent repeated measures and to test for the effect of site on symptom development. QIC criterion was used to evaluate model selection.

2012: Project Manager, School of Public Health and Information Sciences, Department of Epidemiology and Population Health, University of Louisville.
Supervisor: Frank D. Groves, M.D., M.P.H.

Employed MedlineR (a library of literature mining alogorithms in R), PubMatrix, and Bitola to mine academic databases to curate a comprehensive literature on tumor immunology. Methodological algorithms were developed and articles were scored and sorted by scientific strength using SAS Enterprise Miner. A formal literature review was produced to update the field on emerging concepts and techniques.

2011: Project Collaborator, School of Public Health and Information Sciences, Department of Epidemiology and Population Health, University of Louisville.
Supervisor: John Myers, Ph.D.

Evaluated the performance of two novel bioassays for the detection of bovine paratuberculosis by employing ROC/AUC analysis. Sensitivity and specificity for each test were calculated, followed by the statistical comparison of each AUC. Estimates and standard errors from each test were utilized to derive a difference score. Difference scores and standard error of the combined tests were then used to calculate z-scores for each respective test. Z-scores were used to obtain p-values to test the null hypothesis that the tests exhibited similar performance.

2011: Project Manager, School of Public Health and Information Sciences, Department of Epidemiology and Population Health, University of Louisville.
Supervisor: Frank D. Groves, M.D., M.P.H.,

Employed MedlineR, PubMatrix, Bitola, and CoPub to mine academic databases to curate a comprehensive literature focused on the association between iron deficiency/iron overload and subsequent development of cancer. Using Python, natural language processing was further applied to published abstracts to extract novel associations between the explanatory variables and outcome. Methodological algorithms were developed and articles were scored and sorted by scientific strength using SAS Enterprise Miner. A formal literature review was then produced to update the field on emerging concepts and techniques.

2011: Project Manager, School of Public Health and Information Sciences, Department of Epidemiology and Population Health, University of Louisville.
Supervisor: Susan Muldoon, Ph.D.

Following a comprehensive Medline literature review, designed and conducted a meta-analysis using SAS software to examine the association between dietary iron excess and subsequent development of Alzheimer's Disease. A weighting variable was created to account for strength of study methodology and statistical analysis. Studies were scored and sorted by employing SAS Enterprise Miner. Effect estimates were derived by applying additivity of chi-squared statistics, creating a composite chi-squared test statistic for all studies using study as the effect. Covariates were then added to the model.

2010-2011: Project Manager, School of Public Health and Information Sciences, Department of Epidemiology and Population Health, University of Louisville.
Supervisor: Carlton Hornung, Ph.D.

Following a comprehensive Medline literature review, designed and conducted a meta-analysis using SAS examining the association between maternal NIDDM and congenital heart defects in neonates. A weighting variable was created to account for strength of study methodology and statistical analysis. Studies were scored and sorted by employing SAS Enterprise Miner. Effect estimates were derived by applying additivity of chi-squared statistics, creating a composite chi-squared test statistic for all studies using study as the effect. Covariates were then added to the model and forest plots were constructed to visually depict study effect.

2009-2010: Project Manager, Health Behavior Change Research Program, Department of Psychological and Brain Sciences, University of Louisville.
Supervisor: Barbara A. Stetson, Ph.D.

Secondary data analysis of 65 adults with Type 1 diabetes examining personal history of hypoglycemia and hypoglycemia-associated cognitions and their associations with physical activity. ANOVA tests were employed to examine the association between participant questionnaire responses and outcome. Self-reported scores on the Exercise Benefits/Barriers Scale (EBBS) were associated with the Stanford Usual Activity Questionnaire (SUAQ) [$R^2 = 0.87$, $F = 3.99$, df = 1,42 $p = 0.042$]. Entry of a 2nd block with the item "disliking sensations of physical activity" yielded no significant change in R^2. Entry of a 2nd block item "difficulty distinguishing sensations of physical activity from low blood glucose" significantly increased R^2 for SUAQ vigorous activity (p=0.049) and marginally increased R^2 for SUAQ moderate activity (p=0.052). Noteworthy in this study was that less than 1/3 of all participants reported receiving physical activity instructions from their health care provider.

2009-2010: Research Coordinator and Project Manager for multi-site study examining psychological predictors for exercise initiation among adults with Type 2 diabetes following a diabetes self-management education program.
Supervisor: Jason Bonner, Ph.D.

General Role: In-field data collection of survey data, participant recruitment, database and supplies management, participant tracking, collaboration on statistical analyses, and expediting data summaries to lead investigator.

Statistical role: employing ANCOVA to determine if the means of each of the outcome variables in the study were equal across levels of salient predictor variables, controlling for other covariates. Employing hierarchical cluster analysis to the Health Action Process Approach model to examine the effects of germane covariates).

Statistical Consultation

2012 Statistical consultant

Assisted in an exploratory factor analysis of a 26-item quality of life survey for a population of adults with type 2 diabetes. Factors were extracted using principal components analysis with varimax rotation and a scree plot was developed. KMO test was employed to verify sampling adequacy (KMO = .93). Three questionnaire components emerged which had eigenvalues above Kaiser's criterion of 1 and in combination explained 55.46% of the variance.

Client: Theresa Jackson, Ph.D. in Nursing, Marshall University

2011 Statistical consultant

Co-developed project methodology and headed statistical testing of a secondary data analysis examining the association between genetic variation in BRCA1/BRCA2 and response to letrozole in cohort of patients with ovarian cancer. This gene-drug interaction project tested the hypothesis that early-stage ovarian cancer patients with BRCA1/BRCA2 mutations would be more responsive to therapy. T-tests were employed to quantify differential demographic characteristics with continuous outcomes and chi-squared tests were employed to examine differential demographic characteristics with categorical outcomes. MANOVA was employed to model differences in treatment response and stage of ovarian cancer as a function of BRCA1/BRCA2 carrier status (i.e., homozygous, carrier, null). MANOVA was followed with discriminant function analysis to obtain the relative contribution of genetic variation to treatment response and stage of cancer.

Client: R. Brad Baker, D.O. OB-GYN, University of Kentucky

Publications

2015: Altar CA, **Carhart JM**, Allen JD, Hall-Flavin D, Winner JG, Dechairo BM. Clinical Utility of combinatorial pharmacogenomics-guided antidepressant therapy: evidence from three clinical studies. Mol Neuropsychiat, 2015

2015: Winner JG, **Carhart JM**, Altar CA, Goldfarb S Lavezzari G, Parsons KK, Marshak AG, Garavaglia A, Dechario BM. Combinatorial pharmacogenomic guidance for psychiatric medications reduces overall pharmacy costs in a 1 year prospective evaluation. Curr Med Res Opin,

2015: Altar CA, **Carhart JM**, Allen JD, Hall-Flavin DK, DeChairo BM, Winner JG. Clinical validity: combinatorial pharmacogenomics predicts antidepressant responses and healthcare utilizations better than single gene phenotypes. The Pharmacogenomics Journal, 2015.

2013: Hall-Flavin DK, Winner JG, Allen JD, **Carhart JM**, Proctor B, Snyder KA, Drews MS, Eisterhold LL, Geske J, Mrazek DA. Utility of integrated pharmacogenomics testing to support the treatment of major depressive disorder in a psychiatric outpatient setting. *Pharmacogenetics & Genomics*, 2013;23(10): 535-548.

2013: Winner JG, **Carhart JM**, Altar CA, Allen JD, Dechairo BM. A prospective, randomized, double-blind study assessing the clinical impact of integrated pharmacogenomics testing for major depressive disorder. *Discovery Medicine*, 2013;16(89): 219-227.

2011: Stetson, B., Bonner, J. **Carhart, J**. (2011). Psychological predictors of self-reported physical activity initiation in type 2 diabetes patients following diabetes self-management education. *Annals of Behavioral Medicine*, 41, S243.

Poster Presentations

2013: Winner, J., **Carhart, JM**., Allen, J., Dechario, B. Psychiatric pharmacogenomic decision support: metal analysis of prospective studies shows improved antidepressant efficacy and predicted outcomes. CINP: Pharmacogenomics and Personalized Medicine in Psychiatry.

2013: Mrazek, D., Altar, CA., Allen, J., **Carhart, JM.** Combinatorial pharmacogenomics testing improves prediction of antidepressant responses and health utilizations compared to single gene testing. American Psychiatric Association.

2013: Hall-Flavin, D., Winner, J., Allen, J., **Carhart, JM.** Translational pharmacogenomics: physician use and satisfaction with an integrated, multi-gene psychiatric pharmacogenomics test and interpretive report. American Psychiatric Assocation.

2011: **Carhart, J.,** Stetson, B., Mokshagundam S. (April, 2011). Hypoglycemia Experience and Barrier Associations with Physical Activity in Type 1 Diabetes. Rapid Communication Presentation at the 32nd Annual Meeting of the Society of the Behavioral Medicine, Washington DC.

Professional Recognition

2012 Nominated as JCTC faculty for Kentuckiana Metroversity Adult Learner Award.

Volunteer and Community Service Experiences

2016 Little Loomhouse's Friendly Fenley Cemetery Cleanup
Cleaned above cemetery of detritus with fellow members of Humana's Clinical Analytics Attribution team

2011-present Carroll County School System Volunteer/Worker
Designed and conducted physics demonstrations for grades K-3 for outdoor classroom

2007-2009 American Cancer Society Volunteer/Worker
Co-organized relay for life fundraiser within a rural community setting

2008-2009 American Diabetes Association Volunteer/Worker
Co-organized fund raising bicycle ride within a rural community setting

2008 Kentucky Humane Society Volunteer/Worker
Conducted measures to curb feral cat population expansion

2005 Habitat for Humanity Volunteer/Worker
Participated in a home rebuild post-flooding within a rural community