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Models to assess food iron bioavailability

by

Seth Mensah Armah

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Nutritional Sciences (Human Nutrition)

Program of Study Committee:
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Ames, Iowa

2014

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LIST OF ABBREVIATIONS

AI	Adequate Intake
ALAS2	delta-Aminolevulinate Synthase 2
AOAC	Association of Official Analytical Chemist
AUC	Area under the curve
BMP	Bone morphogenic proteins
CDAI	Congenital dyserythropoietic anemia type 1
CDF	Cumulative distribution function
CREBH	Cyclic -AMP responsive element binding protein H
CRP	C-reactive protein
DCT1	Divalent cation transporter I
DCYTB	Duodenal cytochrome B
DMT1	Divalent metal transporter I
dNTP	Deoxyribonucleotide triphosphates
DRI	Dietary Reference Intake
EAR	Estimated Average Requirement
EPO	Erythropoietin
FNDDS	Food and Nutrient Database for Dietary Studies
FPN1	Ferroportin
HAMP	Hepcidin antimicrobial peptide
HCP1	Heme carrier protein
HJV	Hemojuvelin
HO-1	Hemeoxygenase 1
IRE	Iron responsive elements
IRP	Iron regulatory proteins
LBW	Low birth weight
LPS	Lipopolysaccharide

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MPED	MyPyramid Equivalents Database
NCI	National Cancer Institute
NHANES	National Health and Nutrition Examination Survey
NRC	National Research Council
PSU	Primary sampling units
RDA	Recommended Dietary Allowance
RGM	Repulsive guidance molecule
RNR	Ribonucleotide reductase
SLC40A1	Solute carrier family 40 member 1
TCA	Tricarboxylic acid
TfR	Transferrin receptor
TIBC	Total iron binding capacity
UL	Tolerable Upper Intake Level
UTR	Untranslated region
WHO	World Health Organization

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ABSTRACT

Iron deficiency is the most common global nutritional problem, which can be attributed mainly due to poor dietary iron bioavailability. Although many methods exist in assessing bioavailability, they may not be applicable for large populations. Algorithms have therefore been developed from single meal studies for assessing iron bioavailability. However, based on exaggerated effect of dietary factors on iron absorption, new algorithms based on complete diet studies are needed. The objectives of these studies were to: (i) develop a new algorithm from complete diet studies data (manuscript 1); (ii) estimate iron absorption from the US diet using the new algorithm (manuscript 2); and (iii) investigate the effect of long-term phytate consumption on iron absorption (manuscript 3). We developed the algorithm using data from four complete diet studies in which nonheme iron absorption was measured in each subject for three different dietary periods. In estimating iron absorption from the US diet, we used the National Health and Nutrition Examination Survey (NHANES, 2001-2002), MyPyramid Equivalents Database (MPED), and the Food and Nutrient Database for Dietary Studies (FNDDS). In the third study, iron absorption from a high phytate test meal was measured using the area under the curve (AUC) for serum iron in female subjects with ferritin < 30 µg/L (n=28) before and after an eight week dietary modification with high (n=14) or low (n=14) phytate diets. In the first study, serum ferritin explained 35 % of the variability in iron absorption, whereas the effect of dietary factors was small. In the second study, iron bioavailability from the US diet was 15% compared to the currently used value of 18 %. The third study found a significant increase in absorption in the high phytate group (640 to 905 µmol/L; $P < 0.05$) and a non-significant decrease (337 to 267 µmol/L) in the low

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phytate group, indicating that the inhibitory effect of phytate on nonheme iron absorption is dampened among individuals who consume high phytate diet regularly. The findings of these studies have implications for iron nutrition policies for setting recommendations for iron intake and biofortification of high phytate staples with iron.

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CHAPTER 1. GENERAL INTRODUCTION

Background

Iron deficiency is the world's most common nutritional deficiency affecting over 3.5 billion people in developing countries alone (1). It refers to a state in which iron stores are depleted and is mainly caused by either poor dietary iron intake or poor dietary iron bioavailability. Iron bioavailability is defined as the fraction of an ingested nutrient that is absorbed and subsequently utilized for normal physiological functions (2). Total iron bioavailability is a composite estimate made up heme and nonheme iron absorption. Heme iron absorption is fairly constant (approximately 25 %) (3, 4), whereas nonheme iron absorption is poor and highly variable (5-8). This is mainly because of the influence of various dietary factors on nonheme iron absorption. The dietary factors that enhance nonheme iron absorption are ascorbic acid and animal tissue. Inhibitors on the other hand are mainly polyphenols, calcium salts, and phytic acid (9).

There are various methods for assessing nonheme iron absorption. In humans it is estimated using radio or stable isotopes of iron. Iron absorption in humans could also be measured using the area under the curve (AUC) for serum iron (10). In large population level studies however, these methods are not feasible due to cost and researcher/subject burden. For example, to estimate iron bioavailability from the United States diet, it was practically impossible to measure absorption by means of a large population studies. For this reason a total iron absorption value of 18 % was estimated using data for 15 subjects from a single study (11). Although this was a reasonable approach, it has a limitation of small sample size. As means of estimating iron absorption in large population studies therefore, several statistical models have been developed which require information on

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the dietary intakes and or iron status data of subjects. These models have been reviewed by Reddy (12). These models were based on data from single meal studies and come with the limitation of not accurately estimating iron absorption from complete diets. Single meal studies are those in which food labeled with an isotope of iron is fed with a meal after an overnight fast. On the contrary, a complete diet study is one in which the main meals of the day are labeled over a period of days. When Hunt (13) used the existing models to estimate iron absorption from published complete diet data, most algorithms either under or overestimated with wide margins. Cook et al. (14) also showed, single meal studies exaggerate the inhibiting and enhancing effects of dietary factors on nonheme iron absorption compared to complete diet. One of the possible explanations of the discrepancy between the single meal and complete diet studies is that with regular consumption, individuals may adapt to the effect of dietary factors on iron absorption leading to a dampening of the inhibitory/enhancing effects of dietary factors (8). It is therefore imperative that a statistical model that is meant to predict iron absorption from complete diets should be developed from complete diet data. This research was therefore conducted to develop a new algorithm using complete diet data as well as to investigate the concept of adaptation in iron absorption.

Objectives

1. To develop a new algorithm for predicting nonheme iron absorption using data from published complete diet studies.
2. To estimate iron bioavailability from the US diet using the complete diet based algorithm.

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3. To determine the effect of long term phytate consumption on nonheme iron absorption.

Thesis Organization

This thesis is organized into six chapters, namely a general introduction (Chapter 1), literature review (Chapter 2), three research manuscripts (Chapters 3, 4 and 5), and a general conclusion (chapter 6). The general introduction comprises of background information linking the three different research studies. The literature review is a comprehensive review on iron bioavailability. The three manuscripts have been prepared according to the *Journal of Nutrition* format. One of the manuscripts (Chapter 3) has already been published, one has been submitted to the Journal of Nutrition (Chapter 5) and one (Chapter 4) is yet to be submitted. Tables and figures in each chapter are placed at the end of the chapter. All references are cited according to the *Journal of Nutrition* format. For each chapter, the references have been placed at the end of the chapter.

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CHAPTER 2. LITERATURE REVIEW

Iron is an indispensable micronutrient in humans. It is required for several physiological function including, hemoglobin synthesis, DNA and RNA synthesis, cellular respiration, cell proliferation and regulation of post-transcriptional gene expression for different proteins (1). The biochemical significance of iron is tied to its ability to exist in two different oxidation states (Ferous (II) and Ferric (III), which makes it able to both accept and donate electrons. The adult human has 3-5 g iron in the body (2), with men having slightly higher (approximately 50 mg/kg) iron content than women (approximately 40 mg/kg) due to the smaller red cells, muscles and liver mass in women (3). Approximately 10 % of the iron in the body is found in myoglobin and iron containing enzymes, and 20-30 % is stored as ferritin and hemosiderin while the remaining 60 to 70 % is found in red blood cells as part of hemoglobin (1). A very small amount of iron is lost from the body owing to the lack of a structured excretory system for iron. This is in the order of 1-2 mg daily and is compensated for by dietary iron absorption. This minimal iron loss occurs through various routes including sweating, skin desquamation, urinary excretion and sometimes bleeding (4). Most iron loss occurs through the gastrointestinal tract (5).

Iron metabolism in the body including iron absorption, transport and storage is tightly regulated. This is essential because excess free iron in the body can lead to free radical generation through the Fenton reaction which damages lipid membranes, proteins and nucleic acids and is implicated in many chronic diseases including neurological diseases (6). Also, the deficiency of iron leads to impaired function of the various iron-requiring enzymes and systems, with consequences such as fatigue, poor cognition and

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low productivity. This review addresses current knowledge on essential concepts in iron metabolism including absorption, transport, storage, utilization and recycling. It also discusses iron bioavailability and iron nutritional status assessment. Sections of this review are also given to assessment of the iron nutritional status in populations including biochemical indicators as well as statistical methods, particularly the probability approach for assessing adequacy of iron intake in populations.

Iron Absorption

Dietary iron comes in the form of heme and nonheme iron. Heme iron is exclusively from animal sources, mainly from the breakdown of hemoglobin and myoglobin (7). Approximately 40 % of iron from animal sources is heme iron (8). Nonheme iron on the other hand is from both plant and animal sources. It is estimated that 85-90 % of iron intake in the United States is nonheme (9, 10). In developing countries, dietary iron is essentially nonheme with limited heme iron intake. For example, among Moroccan children 6 to 10 years old, Zimmermann et al. (11) estimated that nonheme iron contributed 97 % of total iron intake. Although nonheme iron constitutes the larger proportion of dietary iron intake, it is less bioavailable than heme iron. Approximately 25 % of heme iron is absorbed, whereas the absorption of nonheme iron can range from <1 % to over 20 % (12). Heme and nonheme iron are absorbed through different pathways, with absorption mostly occurring in the duodenum. Iron from nonheme sources is in the ferric form and must be converted into ferrous iron before absorption. This is facilitated by the ferric reductase duodenal cytochrome b (DCYTB) (6). Iron enters the enterocytes through the apical membrane. The uptake of nonheme iron is mediated by divalent metal transporter I (DMT1) also known as divalent cation

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transporter I (DCT1). Heme iron on the other hand is in the ferrous form. It is transported across the apical membrane by a mechanism that is still not well known. It was suggested to be transported by heme carrier protein (HCP1), however, HCP1 has been shown to transport mainly folate (13). Heme enters the enterocyte as intact iron protoporphyrin complex (4). Inside the enterocyte, the iron is released from heme by the hemeoxygenase 1 (HO-1).

Once inside the enterocytes, both sources of iron enter a common labile iron pool (7). The iron is either stored as ferritin or exported out of the enterocyte by ferrotportin (FPN1) (1, 4). Most of the excess iron that is stored as ferritin is lost through exfoliation of the cell. Only about 10 % of the iron stored in ferritin is later exported out of the enterocyte (14). FPN1 is expressed in the basolateral membrane of the enterocytes and in macrophages, liver, placenta and spleen (1). Surface expression of FPN1 is controlled by hepcidin, a peptide hormone which also regulates its function by triggering its internalization and degradation in response to iron replete conditions (4). Iron exported by FPN1 is taken up by the transport protein transferrin. Due to ferroxidase activity, the ferrous iron exported by FPN1 is converted to ferric iron and transported by transferrin. In the enterocytes, the ferroxidase activity is provided by hephaestin, a homologue of ceruloplasmin. In non-intestinal cells, the ferroxidase activity is provided by ceruloplasmin. The expression of the mRNA for DCYTB, DMT1, HO-1 and FPN1 is regulated by hypoxia inducible factor 2 (HIF-2), while posttranscriptional regulation of DMT1 and FPN1 is by iron regulatory proteins (IRPs) in response to intracellular iron levels (4, 15).

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Iron Transport and Cellular Uptake

The ferric iron is taken up by transferrin and transported to target cells. Transferrin is an 80kDa glycoprotein, synthesized mainly by the liver, that has two domains for binding iron molecules (2). It is synthesized mainly by the liver and binds iron with very high affinity at pH of 7.4 (2, 16). The holotransferrin is taken up into the cell by transferrin receptor (TfR), a transmembrane protein consisting of two identical subunits each with a molecular weight of approximately 90-95kDa. Each of the two subunits of the TfR binds to one transferrin molecule. There are two types of TfR, namely TfR1 and TfR2. TfR1 is expressed in all tissues and is synthesized in the endoplasmic reticulum, while TfR2 is expressed only in hepatocytes, duodenal crypt cells and erythroid cells (2, 6). At the target cell surface, TfR1 binds to the holotransferrin and the complex is internalized through endocytosis. The release of the iron is mediated by acidification of the endosome by ATPase proton pump. The iron is released at a pH of ~5.5. DMT1 transports the iron across the membrane of the endosome into the cytoplasm (17). It is then stored as ferritin or utilized. The apo-transferrin-TfR1 complex returns to the cell surface to be able to bind new transferrin. TfR2 binds to transferrin with a lower affinity than TfR1and a mutation in its gene has been linked to hemochromatosis (6, 18).

Iron Storage

The body stores 0-15 mg/kg of iron mainly as ferritin with a small proportion as hemosiderin. Over one half of the body's ferritin is found in the liver, whereas the remaining is mainly found in muscles and in the reticuloendothelial system. Ferritin is composed of 24 subunits and a cavity capable of storing over 4,000 iron atoms in the ferric form (18, 19). Mammalian ferritin comes in two different subunits, namely the

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heavy (H) and the light (L) chain subunits. The H-ferritin is a 22 kDa protein whereas the L-ferritin is a 20 kDa protein. The two isoforms contain 182 and 174 amino acids respectively. Ferritin is approximately 20 % saturated in vivo which implies that approximately 800 iron (Fe III) sites are filled (5). The proportion of the two subunits is dependent on the type of tissue (6). Ferritin with high level of the H subunit is mostly found in the heart and brain, and has high ferroxidase activity, whereas, the L dominating ferritin is found predominantly in the liver and spleen with more iron storage capacity in their cavity, and facilitates iron nucleation and turnover of the ferroxidase center (19, 20). Ferritin first reacts with ferrous iron, oxidizing it to ferric iron for deposition into its cavity. It uses dioxygen as an oxidant in the reaction that occurs at the ferroxidation center. Since this reaction uses both ferrous iron and oxygen, it is considered inhibitory to the formation of free radicals. Thus apart from iron storage, ferritin also deprives the fenton reaction of important substrates. Iron stored in ferritin is released when needed by the body, mainly through proteolytic ferritin degradation. Alternatively, in disease conditions, ferric iron in ferritin may be reduced to ferrous iron resulting in the release of the iron (19). Most of the stored iron in the liver is stored as ferritin, with approximately 5 % stored as hemosiderin. Hemosiderin, found mainly in kupfer cells lysosomal remnant, is a byproduct of lysosomal degradation of ferritin, particularly H-rich ferritin. It is reported that in iron overload, there is increased accumulation of both hemosiderin and ferritin irrespective of the cause of the iron overload. However, the rate of accumulation of hemosiderin is higher (about 10 times that for ferritin) (5, 21). This may be due to that fact that ferritin is damaged during iron overload by increased oxidation

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reduction reactions, leading to the formation of more hemosiderin (or damaged ferritin) (22).

Regulation of Iron Storage and Uptake

Ferritin and transferrin receptor

Regulation of iron storage and uptake occurs mainly at the post-transcription/translation level. This is accomplished through the iron responsive elements (IRE) and IRPs. There are two types of IRPs, namely IRP1 and IRP2. Both types of IRP are expressed in all tissues, however, IRP2 is expressed in lesser quantities in most tissues. The function of this protein depends on the intracellular iron concentration. The IREs are located either at the 3' untranslated region (UTR) or the 5'UTR of the mRNA. They may act as either repressors/inhibitors or enhancers of translation under different condition. For proteins with IRE on the 3'UTR, the binding of the IRP to the IRE leads to the stability of the mRNA resulting in translation and increased synthesis of the protein. On the other hand when the IRE is located at the 5'UTR, the binding of the IRPs to the IRE blocks translation of the mRNA (20). While the IRE for ferritin is located at the 5'UTR that for TfR is located at the 3' UTR. In iron deficiency, the IRP binds to the IRE for both ferritin and TfR. In the case of ferritin, this results in the blocking of translation and thus prevents ferritin synthesis. On the other hand, there is increased stability of the TfR mRNA, resulting in translation and increased protein synthesis. On the contrary, in iron replete situation IRP does not bind to the IRE, there is increased translation of the ferritin mRNA and increased ferritin synthesis for iron storage. TfR mRNA on the other hand is rendered unstable, affecting the translation and synthesis of the protein. Iron regulates the function of IRP1, which is also capable of functioning as an isoform of

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cytoplasmic aconitase. In iron replete conditions, there is the assembly of [4Fe-4S] clusters which reduces the affinity of IRP1 for the IRE. In iron deficiency conditions however, there is a disassembly of the [4Fe-4S] cluster. Thus IRP1 has a strong affinity for the IRE. It therefore binds IRE, functioning as a DNA binding protein. IRP2 unlike IRP 1 does not have [4Fe-4S] clusters. Its regulation by iron occurs through degradation by proteasomes (23).

Apart from its role in the storage of iron and the prevention of free radical formation, ferritin is a major predictor of iron absorption from meals. It has been reported that ferritin accounts for at least 60 % of variability in iron absorption (from nonheme sources) affecting absorption by 10-15 folds (24). Other authors have suggested a more moderate influence of ferritin on nonhene iron absorption. According to Armah et al. (25) ferritin explains 35 % of variability in nonheme iron absorption from whole diets and Reddy et al. (26) reported that ferritin accounted for 32 % of variability in nonheme iron absorption. Ferritin is inversely related to iron absorption. As iron stores increases, the absorption of nonheme iron decreases and vice versa (27).

The role of hepcidin in iron metabolism

Hepcidin is an antimicrobial hormonal peptide, considered as the main regulator of iron absorption. It is important in iron homeostasis playing a key role in iron absorption, recycling and mobilization (28). Hepcidin is composed of 25-amino acids, and is mainly produced by the liver. The liver hepatocytes produce preprohepcidin, the precursor form. This is encoded by the hepcidin antimicrobial peptide (HAMP) gene. The preprohepcidin consists of 84 amino acids, and is converted into the 25 amino acid hepcidin through enzymatic cleavages. Apart from hepcidin 25, other isoforms of

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hepcidin (22 and 20 carboxyterminal amino acid forms) are also produced, however these have little or no biological activity (28, 29).

HAMP gene transcription is induced by various factors including body iron stores, erythropoiesis, oxidative stress, hypoxia and inflammation (29, 30). High body iron stores and inflammation increase hepcidin concentration whereas hypoxia and erythropoiesis downregulate hepcidin production. Among the different factors, body iron store is the main inducer of hepcidin expression. The activation of the HAMP gene by iron status is effected through the bone morphogenic proteins (BMP) and their receptors at the plasma membrane of hepatocytes. Repulsive guidance molecules (RGM) control the downstream effect of the BMP. An example of RGM is hemojuvelin (HJV) which provides specificity to the iron signal in the liver. Other proteins that play important role in the induction of hepcidin expression by iron are HFE and TfR 2. The functional loss of these proteins is associated with reduced hepcidin expression (29). Hypoxia, on the other hand is suggested to decrease hepcidin production by stimulating erythropoietin (EPO) synthesis. The body responds to hypoxia by increasing red blood cells production, a process mediated by hypoxia inducible factors (HIFs) which stimulate EPO production in the liver and kidney, leading to erythropoiesis. Studies have shown that HIF1 suppresses the HAMP gene through this process (31, 32). Due to increased erythropoiesis, there is increased iron requirement, resulting in decreased hepcidin levels and increased iron absorption (4). During infection or inflammation, hepcidin concentration is increased. Hepcidin is an acute-phase protein and the increased production of hepcidin is mediated by lipopolysaccharides (LPS) and cytokines mainly interleukin- 6 (IL-6) (4, 33). LPS on

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the other hand may mediate hepcidin production by inducing endoplasmic reticulum stress and activating C-AMP responsive element binding protein H (CREBH) (29, 34).

Hepcidin regulates iron homeostasis through its regulation of FPN1 expression and function (28). Hepcidin binds to FPN1 to reduce iron export. FPN1, a multi-domain transmembrane protein, is the main exporter of iron from the enterocyte, hepatocytes and macrophages. It is encoded by the solute carrier family 40 member 1 (SLC40A1) gene and expressed by duodenal enterocytes, hepatocytes, reticuloendothelial macrophages as well as placental syncytriotrophoblast. The binding of hepcidin to ferroportin leads to ferroportin being internalized, dephosphorylated and degraded (29). This results in lower iron absorption and impaired release of iron from macrophages.

Hepcidin is implicated in anemia of inflammation and chronic disease, and in hemochromatosis, two virtually opposite conditions, due to its role in regulating iron homeostasis. Anemia of chronic disease, which is linked to overexpression of hepcidin, is characterized by decreased iron absorption in the intestines and macrophage iron release, and an increase in reticuloendothelial iron. The stimulation of hepcidin production in inflammation is attributed to the cytokine IL-6 (35, 36).

On the other hand, hemochromatosis which is mainly caused by insufficient production of hepcidin by the hepatocytes is characterized by inappropriately low levels of hepcidin and hyperabsorption of dietary iron which leads to tissue iron accumulation and consequent iron mediated organ dysfunction (37, 38). In a study to investigate the relationship between hepcidin and different conditions of iron overload, Papanikolaou et al. (39) measured hepcidin levels among 21 subjects with hemochromatosis caused by different conditions. They found that patients with thalassemia had very low urinary

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hepcidin values, with some having no detectable values. Among subjects with congenital dyserythropoietic anemia type 1 (CDAI) and those with Juvenile hemochromatosis hepcidin levels were also undetectable.

Iron Utilization and Recycling

Most intracellular iron metabolism occurs in the mitochondria. However, there is limited data on the mechanism of iron transport from the endosome after cellular uptake to the mitochondria. Mitochondria is the site for the synthesis of Fe-S clusters and heme (40). The synthesized heme is incorporated into hemoglobin and other hemoproteins. Fe-S clusters are protein cofactors found in the mitochondria, nucleus and cytosol that take part in catalytic and regulatory processes in electron transport, tricarboxylic acid (TCA) cycle, amino acid biosynthesis and DNA replication and repair and the regulation of gene expression (41). Fe-S cluster are also critical in the regulation of iron homeostasis through the regulatory binding protein IRP1, which with the acquisition of a 4Fe-4S cluster functions as (c-) aconitase under iron replete conditions (42). The synthesis of heme occurs mainly in developing red blood cells in the marrow with a small fraction (~15 %) occurring in the liver. The biosynthesis of heme occurs in the following four steps: [1] formation of the pyrrole, [2] assembly of the tetrapyrole, [3] modification of the tetrapyrole sidechains, and [4] the formation of protoporphyrin IX and insertion of iron (3). The insertion of iron into protoporphyrin IX is catalyzed by ferrochelatase. The Fe-S clusters produced in the mitochondria are linked to the synthesis of heme through its effect on the translation of the mRNA for delta-aminolevulinate synthase 2 (ALAS2) one of two enzymes that catalyze the formation of the pyrrole in heme biosynthesis (3). In the translation of the ALAS2 mRNA, the binding of IRP to IRE at the 5’ UTR blocks

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translation. However, the addition of an Fe-S cluster reduces the affinity of the IRP for IRE inhibiting the binding and enhancing the translation process (3).

Iron recycling is a primary function of macrophages which are commonly found in bone marrow and spleen. They catabolize heme from senescent and damaged erythrocytes releasing the iron using heme oxygenase after phagocytosis. Macrophages recover the iron for the synthesis of hemoglobin and for incorporation into iron requiring enzymes. Iron recycling contributes significantly to daily iron needs. About 20 mg of iron is needed for *de novo* hemoglobin synthesis every day, which is mainly obtained from recycled iron (4). The recycling process contributes over 10 -20 times the iron flux from intestinal iron absorption (20, 43).

Functions of Iron

DNA synthesis

The role of iron in DNA synthesis and repair is tied to its role in the activity of the enzyme ribonucleotide reductase (RNR). RNR catalyzes the rate limiting step in the *de novo* synthesis of deoxyribonucleotide triphosphates (dNTP) (44). They reduce ribonucleotides to dNTP. The RNR enzyme is composed two subunits, R1 and R2. The R1 subunit contains the catalytic site for the binding and reduction of ribonucleotides to dNTPs, and two sites for allosteric binding, and is located in the cytosol. The R2 subunit on the other hand is located in the nucleus and contains a tyrosyl group which requires iron for its stability. While the R1subunit is expressed at all stages of the cell cycle, the R2 subunit synthesis is only initiated at the S-phase for DNA synthesis. Impaired iron supply to the R2 inactivates R1 leading to impaired dNTP synthesis with implication on DNA synthesis and repairs (45, 46).

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Iron and energy metabolism

The enzyme aconitase is involved in the interconversion of citrate and isocitrate through a cis-aconitase intermediate as seen in the citric acid cycle and glyoxylate cycle. There are two types of aconitase, which are coded by different genes. These are the cytoplasmic (c-) aconitase and the mitochondrial (m-) aconitase. The iron regulatory protein, IRP1 is bifunctional, acting as a DNA binding protein in iron deficiency and as a (c-) aconitase in iron replete situations. The role of IRP1 depends on the presence or absence of an [4Fe-4S] cluster. In iron replete conditions, there is assembly of [4Fe-4S] clusters in IRP1 which decreases its affinity for the IRE turning it into (c-) aconitase. In iron depleted conditions, the [4Fe-4S] cluster is dissembled, and IRP1 acts as a binding protein (44). The enzymatic activity of (m-) aconitase, which catalyzes the interconversion of citrate and isocitrate in the citric acid cycle, is affected by iron levels. Like the (c-) aconitase, it also contains a [4Fe-4S] cluster, and its function is regulated by the assembly and disassembly of the [4Fe-4S] cluster (47)

Oxygen and electron transport

Iron is critical in the transport of oxygen as well as in the respiratory chain. Many of its functions are related to its importance in heme biosynthesis. Heme is composed of a ferrous iron inserted in the center of a protoporphyrin ring that consists of four pyrrole rings. It is a key component of hemoglobin which is required for the transport of oxygen and carbon dioxide in the cardiovascular system. Hemoglobin, a tetrametric protein composed of four polypeptide chains (2 alpha and 2 beta globin chains), is mainly found in erythrocytes. It carries oxygen from the lungs to the body tissues and carbon dioxide from the tissues to the lungs. Apart from being a component of hemoglobin, heme is also

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a cofactor for many enzyme including catalases and peroxidases. It is also a component of cytochromes, electron transport proteins found mostly in the mitochondria that play important roles related to energy metabolism (48). Apart from cytochromes, the role of iron extends to its function as part of Fe-S clusters. Fe-S clusters also play important roles in the respiratory chain. Fe-S clusters like the cytochromes are involved in electron transport. They also play several other biological roles including substrate binding and activation, regulation of gene expression, regulation of enzyme activity, and disulphide reduction (49).

Iron Deficiency and Anemia

Iron status refers to a continuum of body iron levels ranging from iron deficiency anemia to iron overload. Iron deficiency essentially refers to the lack of mobilizable iron stores due to depletion of body iron stores. The body iron homeostasis is tightly regulated. With no system in place for excreting iron from the body, body iron needs are mostly met by recycling iron. The very little iron (1-2 mg) that is lost daily is replaced by absorbed dietary iron. Typically iron from stores or from the intestines after absorption is transported to target cells by transferrin. When iron supply from these sources are diminished, there is a decrease in iron available for transport by transferrin as well as transferrin saturation, and a concomitant increase in TfR as compensatory mechanism to take up as much iron as possible (50, 51). With time, iron deficiency affects erythropoiesis resulting in a decrease in hemoglobin levels. While iron deficiency contributes a huge proportion of anemia cases, anemia is also caused by other factors. It is estimated that up to 50 % of anemia cases are due to iron deficiency while other causes include intestinal parasites, malaria, and deficiency in other nutrients (vitamin B12,

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folate, etc.) as well as congenital issues (51, 52). Anemia is a highly prevalent public health concern around the globe. It affects over 1.6 billion people the world over and mostly common in developing countries (52). It is estimated that some 46 % of people in Africa and 56 % of South-East Asians are anemic (53). Most affected groups are women of child bearing age (due to menstrual losses), and infant, young children and pregnant women (as a result of their increased iron requirements) (54). Iron deficiency is mostly attributed to poor iron absorption, low dietary iron intake, increased iron requirement and increased iron loses (8). Particularly in developing countries, poor iron absorption is considered one of the key causes of iron deficiency. In these countries, dietary iron is mainly from nonheme sources which are not well absorbed. The diets in these countries are also high in phytic acid which is a known inhibitor of iron absorption. Another component of the meal that may inhibit nonheme iron absorption in developing countries is polyphenol which occur in high levels in some legume varieties such as beans consumed frequently in developing countries as well as tea and coffee.

Consequences of Iron Deficiency

The consequences of iron deficiency anemia are grave including adverse pregnancy outcomes, poor cognitive development, reduced working capacity and productivity, and impaired endocrine and neurotransmitter function. It is also associated with decreased resistance to infections, increased heavy metal absorption, and increased infant mortality (55). The following sections discuss some of the consequences of iron deficiency.

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Adverse pregnancy outcome

Pregnant women are the most vulnerable groups for iron deficiency and anemia, especially during the last trimester. This happens because most iron transfer to fetus occurs after 30 weeks of gestation. Pregnancy is associated with marked changes in hematological parameters. For instance, there is an increase in plasma volume and a consequent decrease in hemoglobin concentration. Also ferritin levels decrease markedly between weeks 12 and 25 (56). Similarly, iron requirement are increased during pregnancy. At the early stages, there is about a 2.5 fold increase in requirement, which later on increases to about 6.5 fold during the third trimester. However, this is accompanied by a concomitant increase in nonheme iron absorption to up to 66 % at 36 weeks of gestation (57). The pregnant mother with poor dietary iron intake becomes prone to iron deficiency particularly in the third trimester because the fetus will rely on the mother's iron stores. One of the key factors relevant for a positive pregnancy outcome is adequate plasma volume expansion. Impairment in plasma volume expansion may result in high blood viscosity, high pressure of blood flow, and placental abruption. There is also increased risk of preeclampsia and intrauterine growth restriction with high hemoglobin concentrations in pregnancy (57). In a study to probe the relationship between maternal hemoglobin concentration during labor and neonatal birth Sekhavat et al. (58) found a significantly increased risk of low birth weight among women with hemoglobin concentrations less than 10 g/dL. Although they observed an increased risk among women with hemoglobin concentrations above 13 g/dl this was not statistically significant. In another study, the relationship between maternal hematological status and risk of low birth weight was investigated among 1,400 Nepalese women in a case-control

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study (59). The authors found that low hematocrit values at first and last trimesters were associated with lowest mean birth weight. They reported that women with severe anemia (Hematocrit ≤ 24 %) had significantly higher risk of low birth weight infant and preterm infant delivery compared to those with hematocrit in the reference range (34-36 %). While one out of every three women in the severely anemic group had a preterm delivery, only 5 % of those in the reference range group had preterm delivery. In another case-control study to investigate the risk factors for low birth weight and preterm births in Ahmedabad, India, Mavalankar et al. (60) found clinical anemia as a significant independent risk factor. In yet another study, Levy et al. (61) found maternal anemia as a risk factor for both low birth weight and preterm delivery.

Poor cognitive development and impaired neurotransmitter function

Iron deficiency is the most prevalent nutrient deficiency between 6-24 months of age. This is the period of maximal brain growth, and unfolding of many neurodevelopmental processes. At birth the brain has reached <30 % of the adult size and continues to grow up to 2 years of age. Since most brain growth occurs at this time, adequate iron in the diet is critical at this stage. The effect of iron deficiency at this stage can only be partially corrected in later life (62). Many studies have shown a relationship between iron deficiency/anemia and poor cognition among children. In a longitudinal study Lozoff et al. (63) followed up with Costa Rican children who had been tested and treated for iron deficiency when they were infants. They found that children (ages 11-14 years) who had severe iron deficiency during infancy were prone to poorer motor and mental functioning compared to their normal counterparts. In another study that investigated the effect of iron deficiency anemia on specific domains of cognitive

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function in infancy, Carter et al. (64) assessed five different aspects of cognitive processing. They found that iron deficiency anemia affected two of the five aspects of cognition, namely object permanence and recognition memory.

A possible explanation for the effect of iron deficiency on cognition is the involvement of iron in the hippocampal formation and neurotransmitter function especially in the dopaminergic system. Another speculated mechanism is related to the fact that iron levels are particularly high in the basal ganglia and the cerebellum that are important in motor function, as well as the role of iron in myelin formation and maintenance (63). The peak period for iron uptake into the brain occurs at the time of myelin synthesis. Overall explanations to the effect of iron deficiency on cognition may be due to either low brain oxygen acquisition or that it leads to low brain iron which results in low neurotransmitter levels, impaired transmission, impaired myelin formation and poor neuromaturation (62, 65, 66).

Reduced work capacity and productivity

Reduced work tolerance and poor working capacity are very common symptoms of anemia. Gardner et al. (67) investigated the relationship between hemoglobin concentration and work capacity and found that work tolerance is lower among anemic subjects compared to subjects with normal hemoglobin concentrations. Edgerton et al. (68) also found a strong positive correlation between hemoglobin concentration and work tolerance among adult subjects. Their work suggested that hemoglobin changes rather than other changes in iron status may largely explain the reduced work capacity in anemia. Among subjects with iron deficiency anemia, Ohira et al. (69) found increased maximal work time after treatment with iron dextran, whereas no changes were found in

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the placebo group. The relationship between anemia and work capacity may be due to the role of iron in energy production. Iron is involved in the transport of oxygen, in the respiratory chain, and is a component of several enzymes. As a component of red blood cells, iron-containing hemoglobin is critical in the transport of oxygen from the lungs to the body tissues. Myoglobin, another iron containing protein in the cytoplasm of muscle cells, is involved in the diffusion of oxygen towards the mitochondria. Cytochromes involved in electron transfer chain in mitochondria and enzymes such as dehydrogenases involved in substrate oxidation all require iron (70).

Assessment of Iron Nutritional Status

Accurate methods for the assessment of iron nutritional status are critical considering the global magnitude of the problem of iron deficiency. The World Health Organization (WHO) (51) recommends that clinical and population level screening for iron deficiency anemia should be based on the resourcefulness of the country in which the assessment is being made. In developed countries, iron status screening at the clinical level should be based on hemoglobin and hematocrit together with serum ferritin and transferrin saturation while diagnosis or confirmation should include other biomarkers such as erythrocyte protoporphyrin, TfR, mean cell volume and response to iron administration. In poorly resourced countries on the other hand clinical examinations (significant pallor of eyelids, palm, nail beds and tongue) should be used in screening to identify high risk individuals while hemoglobin or hematocrit with iron administration is used for diagnostics or confirmation of anemia in individuals. The complexity in the assessment of iron status lies in the fact that each of the different biomarkers has its own limitations. Ferritin is one of the commonly used measures of iron stores. Among

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children under 5 years of age, a ferritin concentration less than 12 µg/L is indicative of depletion in iron stores, while the cut off for older individuals is less than 15 µg/L. However, apart from the fact that ferritin level differ between males and females (higher in males) ferritin is increased during infection because it is an acute phase protein which may be due to the fact that inflammation inhibits iron release from reticuloendothelial stores (71). Thus, ferritin is only a reliable marker of iron stores in populations free of infection and inflammation. Unlike ferritin, TfR levels increase in iron deficiency. This biomarker is also less affected by inflammation and infection and it does not vary by gender, pregnancy status or age, however it is elevated when erythroid activity increases (red blood cell synthesis and turnover). Other iron status biomarkers such as tranfserrin saturation, and total iron binding capacity (TIBC) are equally limited in their usefulness in assessing iron status. In iron deficiency serum iron and transferrin saturation decreases while the TIBC increases. Transferrin saturation and serum iron are affected by diurnal variations (51). A combination of multiple biomarkers is thus commonly used in assessing iron status. For example, in assessing iron deficiency in the US population, Cogswell et al. (72) used two different models, one being the ferritin model which classifies individuals as iron deficient if they had abnormal values in any two of three parameters (serum ferritin, transferrin saturation and erythrocyte protoporphyrin). The ferritin model was also used by Looker et al. (73) in estimating iron deficiency in the US population. The most recommended combination being hemoglobin (functional iron), TfR (tissue need for iron) and ferritin or bone marrow iron (iron stores) (51).

A relatively new marker for iron status is hemoglobin content of reticulocytes (CHr). This measure indicates how much iron was available to be used in red blood cell

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synthesis within the past few days. There are, however, no final cut off values suggested for CHr yet (71). Another biomarker that is useful in assessing iron status is the TfR/ferritin ratio. This marker measures iron deficiency without anemia a stage of iron deficiency also referred to as iron deficiency erythropoiesis. In assessing the usefulness of the different biomarkers in assessing tissue iron deficiency, Skikne et al. (74) reported that ferritin was the most sensitive marker when there are residual iron stores, whereas the TfR is better in functional iron deficiency. In addition, the ratio of the TfR to serum ferritin provides a marker useful in assessing iron status over a wide range including states of ample iron stores to functional iron deficiency.

Strategies to Address Iron Deficiency Anemia

Supplementation

Supplementation is the fastest way to address nutrient deficiency in populations. Several studies have investigated the benefits of iron supplementation in improving iron status and mitigating the adverse consequences of iron deficiency and anemia. Menendez et al. (75) reported lower incidence of severe anemia associated with iron supplementation among infants born in a malaria hyperendemic hospital in Tanzania. Among pregnant women in Niger, Preziosi et al. (76) found a significant decrease in anemia and iron deficiency in the iron supplementation group during the last trimester. Other studies have also reported reduced risk of behavioral problems in children, reduced incidence of low birth weight (LBW) infants, higher birth weight, as well as prevention of iron deficiency anemia among pregnant women due to iron supplementation (77-79).

The WHO strongly recommends intermittent (weekly) iron supplementation for preschool and school aged children in setting with $\leq$20 % prevalence of anemia among

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these age groups. The recommended doses are 25 mg elemental iron for preschool children 24-59 months and 45 mg for school age children (5-12years) (80). Among pregnant women, it is recommended that they receive 60 mg iron supplementation daily for 6 months and among those living in areas with iron deficiency anemia prevalence > 40 %, iron supplementation is recommended to continue to 3 months postpartum. This is to be taken together with 400 µg folic acid/day (81). Sources of elemental iron for iron supplementation include ferrous sulfate, ferrous fumarate or ferrous gluconate (80).

Recently, there have been concerns about the safety of iron supplementation in malaria endemic areas. Among children in malaria endemic areas, malaria is one of the major causes of anemia and malarial anemia accounts for about a third of all deaths from the disease (82). More importantly some authors have suggested that iron supplementation in malaria endemic areas may increase malaria or mortality risk. In a randomized trial conducted in Zanzibar (83) an increased risk of death or hospitalization among children receiving iron and folic acid supplement was found, compared to placebo group and it was concluded that while anemic and iron deficient children may have some benefit from the iron supplementation, iron replete children may find it harmful. However, Ojukwu et al. (84) have concluded in a Cochrane review that with malaria surveillance and treatment services in place, there is no increased risk of malaria or death with iron supplementation in malaria endemic areas.

Fortification

The effect of iron fortified foods on iron status in different populations has been widely studied. A six month intervention study using NaFeEDTA fortified fish sauce resulted in improved iron status biomarkers and reduced iron deficiency anemia

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prevalence among Vietnamese women (85). Other similar studies have also shown a positive impact of iron fortification on iron status (86-88). These studies have used vehicles such as sugar, curry powder, and candy fortified with iron to improve iron status of subjects. While food fortification is not as fast as supplementation in addressing nutrient deficiency, it is an effective approach and its impact is much more sustainable in the long-term. It requires the identification of a food vehicle that is commonly consumed by the target population and has a high bioavailability. Also the food fortificant must not alter the organoleptic properties of the food, must be widely available, cost-effective and must be well absorbed (53, 55).

An alternative form of fortification is biofortification. With biofortification, plants are bred to increase their nutritional value. Few studies have investigated the potential of iron biofortified crop in improving iron nutritional status (89, 90). In one such study among Filipino nuns, the investigators found that the biofortified rice significantly improved ferritin and body iron levels among subjects who were nonanemic (89). Biofortication has the advantage of a low recurrent cost. It is also a sustainable approach to addressing iron deficiency (91). Also since it is usually done with staple crops, the product has a high chance of being predominantly consumed by the poor and people most vulnerable to nutritional deficiencies. In addition crops that are biofortified to become nutrient dense have the added advantage of been more resistant to diseases. A major challenge in the use of biofortification to address micronutrient deficiency in general is the acceptance of the new variety, especially where there are visible changes in appearance characteristics such as color of the staple (92).

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Food based approach

Apart from supplementation and food fortification, iron nutritional status in a population can also be improved through food based approach. While iron supplementation represents a short-to-medium term approach to addressing iron deficiency and anemia, food fortification and dietary diversification represent medium-to-long term approaches (93, 94). Patterson et al. (94) compared the effect of a dietary treatment with iron supplementation in improving iron status among iron deficient women of childbearing age. The subjects were randomized to either receive 105 mg/d of inorganic iron from ferrous sulfate, or an iron rich diet with 2.25 mg/d of absorbable iron for 12 weeks. They found that although the iron supplementation improved ferritin levels faster (from 9 to 25 µg/L) than the diet (from 9 to 11.0 µg/l), the ferritin level in the high iron diet group continued to increase during the 6 month follow up period. Verrall et al. (95) investigated a food-based approach to improve iron nutritional status among Canadian infants. They conducted repeated cross sectional surveys at two different time points to evaluate the impact of an intervention involving the dissemination of information aimed at promoting the use of optimal iron-rich complementary foods. They used posters, newsletter articles, pamphlets and homemade infant food cooking activities as communicating strategies. They found a significant increase in iron intake from complementary foods between the two time points.

Typically, increasing overall food intake is associated with an increased intake of iron as well as other nutrients. This is particularly important where overall food intake or food security is poor. In such cases, multiple micronutrient deficiencies may also co-exist and thus ensuring adequate food availability should be a priority (96). Increasing the

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intake of iron rich foods is key in addressing iron deficiency in particular. The bioavailability of the iron from the food is of utmost importance in addressing iron deficiency. In countries where iron deficiency prevalence is high, poor bioavailability is considered one of the major causes of iron deficiency (97). To ensure the adequacy of iron intake, animal source foods, as well as plant based iron consumed together with fruits and vegetables rich in vitamin C must be regular components of the diet. Dietary inhibitors of iron absorption must not be consumed together with the iron containing meal, especially for plant-based diets. Inhibitors of iron absorption include polyphenols from beverages such as tea and coffee, phytic acid which is mostly found in whole grain legumes and nuts, and calcium salts. Strategies to reduce the effect of these inhibitors includes consuming tea at least two hours after consuming the meal, soaking, germinating or fermenting grains to minimize the phytate content, and avoiding the ingestion of calcium supplement or dairy products high in calcium together with the iron containing meal (96, 98).

Estimating Prevalence of Inadequate Iron Intake

Prevalence of inadequate nutrient intake is an estimation of the proportion of the population whose intake of the nutrient is less than their requirement. Over the years various approaches have been used to estimate this prevalence including the use of various proportions of the RDA and other dietary reference values. The RDA is one of several Dietary Reference Intakes (DRIs) and is defined as the average daily dietary intake level, sufficient to meet the nutrient requirements of nearly all (97-98 percent) healthy individuals in a group (99). However, using selected cut-offs based on the RDA (such as 50 %, 66 % or 70 % of RDA) cannot be justified especially when used at the

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population level. Apart from the RDA, the other DRIs are the Estimated Average Requirement (EAR), the Adequate Intake (AI) and the Tolerable Upper Intake Level (UL). Recently, the EAR cut-point method was introduced as a more efficient and unbiased way of assessing prevalence of inadequate nutrients intake in a population (100). In this approach, prevalence of inadequate nutrient intake is estimated as the proportion of individuals whose usual intake level is below the EAR. The EAR is the average daily nutrient intake level estimated to meet the requirements of half of the healthy individuals in a group (99). It is estimated as RDA -2SD (of requirement) (101). Using the EAR cut-point method requires that certain assumptions be met. These are:

I. Intake of and requirement for the nutrient are assumed to be independent;
II. The requirement distribution is symmetrical around the EAR; and
III. The variability in intakes among individuals in the group is greater than the variability in requirements of the individuals.

Also, the actual prevalence of inadequacy should not be too low (below 8-10 %) or too high (90-92 %) (101). It is well-known that the nutrient intake of individuals vary from day to day. Similarly when averaged over time, there are differences in the intakes of these nutrients between individuals. It is important that before assessing the prevalence of inadequate nutrients intake, nutrients intake data is corrected for within person (day-to-day) variability. This is done to give a good estimate of the usual intake distribution of the nutrient in that population. The usual intake is defined as the long-run average of daily intakes of the nutrient or a dietary component by an individual (102). Typically in dietary assessment, food intake data are collected over a number of days in many cases 2 or 3 non-consecutive days including at least a weekday and a weekend. While the mean

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of these intakes may be an unbiased estimator of the usual intake, the variance will not. For this reason the distribution of the daily intakes will not accurately represent the distribution of usual intakes. This is because the daily intake distribution will have a higher variability than the usual intake distribution due to day-to-day variability. This also implies that if you use the mean intake or daily intake distribution to estimate the proportion of individuals with intake above or below a given cut point on the distribution curve, the results may not be accurate since some parameters of the distribution may differ between the mean intake distribution and the usual intake distribution. Nusser et al. (103) proposed a method for estimating usual intake distribution using the method of moments. Without transforming the data, this method estimates the usual distribution using the measurement error model. The moments for the usual intakes are then estimated using method of moments. These are then used to estimate the parameters of the distribution of usual intakes. Another approach for estimating the usual intakes was recommended by the National Research Council (NRC) (104). This method consisted of transforming the data, then fitting the measurement error model to estimate the within and between person variability and then back transforming the data into the original scale. The limitations of the NRC measurement error model includes the fact that transforming and back transforming the data may introduce bias in the estimate of the usual intake. Moreover, log transformation does not necessarily guarantee normality of the data (105). This method was improved upon by Nusser et al. (102) in a method popularly referred to as the Iowa State University (ISU) method. In this approach to estimating usual intake distribution, the data is first cleaned to remove the effect for noise variables such as day of the week. In this step, the intake data is adjusted to the first day

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data. In the second step, survey weights are incorporated into the analysis to account for complex survey designs. The next step involves the transformation of the weight adjusted data using a combination of methods (power transformation, and grafted polynomial function). The data is first transformed using the power transformation and then mapped to the normal scale using the cubic spline transformation (105). Using the transformed data, the parameters of the usual intake distribution are then estimated. Like the previous methods, the ISU method also uses a measurement error approach in estimating the parameters of the usual intake distribution. The estimated usual intake distribution is then back transformed in a manner appropriate to the distribution of individual means (102). Apart from these methods, the National Cancer Institute (NCI) also developed a new approach for estimating usual intake distributions using a mixed effect model. The NCI method uses Box-Cox transformation and covariates in estimating the usual nutrients intakes. The percentiles for the usual intake distribution are estimated using the Monte Carlo simulation method (106). Once the data is corrected for the day-to-day variability, the usual intake data can now be used to estimate the prevalence of inadequate iron intake. Although the EAR-cut point method is a reliable approach for estimating the prevalence of inadequate nutrients intake, it does not work for some nutrients because the assumptions are not met. For example in the case of iron, the second assumption is violated because the requirement distribution is not symmetric around the EAR. Similarly in the case of energy, the intake and requirement distributions are not independent, violating the first assumption.

Thus, in estimating the prevalence of inadequate iron intake, the probability approach is used (107). In the probability approach, each intake level (interval) is

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assigned a probability of inadequacy based on a risk curve, and the prevalence of inadequate nutrient intake is estimated as the weighted average of the risk (probability) values. The risk curve gives the probability that a particular intake level is less than or equal to the requirement and is calculated based on the cumulative distribution function (CDF) of the requirements for that nutrient (100). To estimate the prevalence of inadequate iron intake using this approach (53):

I. The proportion (%) of the population whose iron intake fell within each given risk (probability) of inadequacy is determined.
II. The different risk values are then multiplied by their respective proportions.
III. The products are then summed up to give the prevalence of inadequate intake.

Iron Bioavailability

Iron bioavailability is defined as the proportion of the ingested iron that is absorbed and utilized for normal physiological functions and storage (24). Iron in the diet comes in two different forms, namely heme and nonheme iron. Heme iron comes from animal sources whereas nonheme iron is found in both plant and animal sources. Approximately 40 % of iron from animal sources is heme iron whereas plant source iron are all exclusively nonheme iron. Heme iron is from hemoglobin and myoglobin and is better absorbed than nonheme iron. In adults with adequate iron stores, approximately 25 % of heme iron is absorbed (108). Nonheme iron must first be reduced from the ferric to ferrous by DCYTB and then transported across the apical membrane of the enterocytes

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by DMT1. Heme iron is first transported across the apical membrane by receptor mediated endocytosis and the iron is released from heme by HO-1 (28).

Factors Influencing Iron Bioavailability

Several factors are known to influence the absorption of iron from the diet. Heme iron absorption is more moderately influenced by iron status of the host, but absorption of nonheme iron is strongly influenced by iron status and also affected to some extent by dietary factors. The dietary factors that influence nonheme iron absorption include calcium salts, phytates, ascorbic acid, animal tissue (meat, fish and poultry) and polyphenols (beans, coffee and tea). Apart from these, the amount of nonheme iron in the diet also has an influence on the percentage absorption with less percentage absorption at higher intakes. Ferritin is the most well-known iron status marker that influences iron absorption. Other host factors such as hepcidin, inflammation or infection and even genetic factors may also be important in the variability of iron absorption among individuals. Among the different dietary factors, ascorbic acid and animal tissue (meat, fish and poultry) enhance iron absorption while the others (phytic acid, polyphenols and calcium salts) inhibit iron absorption.

Ascorbic acid

Ascorbic acid is a water soluble vitamin with both enzymatic and non-enzymatic functions. In its enzymatic functions, it serves as a cofactor for many metabolic reactions including hydroxylation of collagen, tyrosine metabolism, carnitine and norepinephrine biosynthesis and peptide hormone amidation. Apart from its enzymatic roles, it serves as a strong antioxidant, helping with the scavenging of free radicals and reactive oxygen species (109, 110). Fruit and vegetable contribute approximately 90 % of vitamin C

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intake with high amounts occurring in citrus fruits, kiwi, mango and broccoli. Deficiency of vitamin C has been attributed mainly to poor dietary intake, and may also be caused by smoking as well as genetic and clinical conditions (109). Most plants and animals synthesize ascorbic acid from D-glucose or D-galactose. Humans, however, do not synthesize ascorbic acid due to the inactivation of the gene that encodes for the enzyme L-gulono-γ-lactone oxidase. Therefore, ascorbic acid must be supplied through the diet (111). Vitamin C is a well-known enhancer of nonheme iron absorption. It enhances nonheme iron absorption by reducing iron from ferric to ferrous form for efficient transport across the apical membrane of the enterocytes. Ascorbic acid is also able to chelate iron to form a complex that is soluble over a wide pH range (112). Its enhancing effect has been shown in various studies (113). When Kuhn et al. (114) investigated the effects of different chelating agents on iron absorption from different foods, they reported an increase in iron absorption from corn and from wheat meals after the addition of ascorbic acid. Callendar and Marney (115) found that the addition of 100 ml orange juice to an egg meal increased iron absorption from 3.7 to 10.4 %. In another study, the addition of papaya containing 66 mg ascorbic acid increased nonheme iron absorption by approximately 5 fold (116). The effect of ascorbic acid on nonheme iron absorption has been shown to be dose-dependent (117). Even in the presence of inhibitors, ascorbic acid is able to counteract the inhibitory effects. In a study by Hallberg et al. (118) addition of 50 or 100 mg ascorbic acid significantly increased iron absorption from meals containing 25 and 250 mg of phytate phosphorus. Ascorbic acid also counteracted the inhibitory effects of polyphenols in a study by Siegenberg et al. (119). For meals containing low to medium level of inhibitors, an ascorbic acid: iron molar ratio of 2:1 is required to

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promote iron absorption, while for those with high amounts of inhibitors, a molar ratio of 4:1 is required (120).

Animal tissue

The pioneering work on the enhancing effect of animal tissue on nonheme iron absorption is attributed to Layrrise and co-workers (1968). Their work investigated the effect of interaction among different food items on iron absorption. They found that adding veal muscle or fish to corn increased iron absorption by about 50 and 300 % respectively. Similarly adding veal muscle tripled iron absorption from black beans. Similarly, when black beans were given with amino acid similar to the combination found in fish, there was approximately three fold increase, suggesting that animal tissue may enhance iron absorption through the amino acids released during digestion. Since then, several other studies have also demonstrated the enhancing effects of animal tissue on iron absorption. For example, Bach et al. (121), have shown that adding meat (≥50g) to a low bioavailability test meal can increase iron bioavailability by over 40 %. Hallberg et al. (122) studied the effect of adding meat (with or without ascorbic acid) to weaning gruel on nonheme iron absorption. They found that the addition of meat powder markedly increased iron absorption from the gruels by 85 % (from 0.33 mg to 0.61 mg). Hallberg and Rossander (123) have also shown that the addition of 75 g meat to low bioavailability meal composed of maize, rice and black beans increased nonheme iron absorption from 0.17 mg to 0.45 mg. Like calcium, animal tissue is also unique in its contribution to iron absorption. The heme content of animal tissue is better absorbed, contributing total iron absorption. Similarly, animal tissue enhances the absorption of nonheme iron in the meal. While the mechanism by which meat enhances nonheme iron absorption is unclear,

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several possible mechanisms have been proposed. For example, the stimulation of gastric juice secretion is one suggested mechanism. When Cook et al. (124) investigated the effect of achylia gastrica on iron absorption, they found that mean iron absorption was lower in the achylic group as compared to the control group, and the addition of gastric juice to the test dose increased iron absorption. Another suggested mechanism is the release of digestion products of animal tissue. Hurrell et al. (125) have suggested that partially digested products form animal tissue are able to bind iron via their histidine and cysteine residues, rendering iron soluble and thus enhancing its absorption. In this way, iron is also unavailable for binding with phytates and polyphenols which might be present in the meal.

Phytic acid

Phytic acid (myoinositol hexakiphosphate, or IP6) is a key inhibitor of iron absorption. It is found in high amount in grains, legumes, nuts and oil seeds. Phytic acid is the main storage form of phosphorus in seeds. It holds up to 80 % of the seed phosphorus (126). The salt form (magnesium, calcium or potassium salt) of phytic acid is referred to as phytate (127). Phytate inhibits iron absorption by binding to the iron to form insoluble complexes that cannot be absorbed at the intestinal pH (128). The negatively charge phosphate in the phytic acid structure binds to metallic cations including iron, calcium and zinc, rendering them insoluble and impairing their bioavailability. In many developing countries where iron deficiency prevalence is high, dietary staples are also known to contain high phytate level of phytate compared to developing countries. For instance, according to Amirabdollahian and Ash (129), median phytate intake among adults in the United Kingdom is 809 mg/day, while the estimated

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phytate intake in Nigerian diet is approximately 2200 mg/day. The average phytate intake among US aldults is estimated to be about 750 mg/day (130).

Mostly, the higher inositol phosphates (myo-inositol hexaphosphate-IP6 and myo-inositol hexaphosphate IP5) are responsible for the inhibitory effects of phytates on iron absorption. In an experiment to determine the effect of inositol phosphates with different number of phosphorus groups on iron absorption, Sandberg et al. (131) found that IP5 inhibited iron absorption but no effect with IP4 and IP3 was observed. In vitro studies have also shown that while even small amounts (0.5 µmol) of IP5 and IP6 reduce iron solubility significantly, IP3 and IP4 did not (132). Like ascorbic acid, the effect of phytic acid on nonheme iron absorption is dose dependent (133).

In high phytate meals, iron absorption can be increased by reducing the phytate content. In a study by Hurrell et al. (134) when phytate contents of soy protein isolates were reduced from 4.9-8.4 mg/g to ≤ 0.01 mg/g by either enzymatic treatment or acid washing and ultrafiltration, iron absorption improved as much as four to five fold. Phytate content of foods can be reduced through soaking, germination, fermentation, and addition of phytase enzyme. During germination, there is an increase in phytase activity leading to hydrolysis of phytate (IP6) into intermediate inositol phosphates (IP-1, IP-2, IP-3, IP-4, and IP-5). This occurs through *de novo* synthesis of the enzyme or activation of endogenous phytase or both (127, 128). In fermentation, the hydrolysis of phytate is due to phytases produced by microflora on the surface of cereal grains. Soaking on the other hand reduces phytate content in most legumes which have phytates stored in water soluble forms such as sodium or potassium phytates (135).

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Polyphenols

Polyphenols are a group of natural compounds with phenolic structural features (136). They constitute the most common dietary source of antioxidants and are known to contributes significantly to human health particularly in the prevention of degenerative diseases like cancer, neurodegenerative diseases, diabetes mellitus, cardiovascular diseases and osteoporosis (137). Polyphenols may act as both antioxidant and prooxidants under different conditions serving different purposes such as improving cell survival and triggering programmed cell death (137). Their health benefits however, depends on their bioavailability and the amount consumed (138). Polyphenols occur in good quantities in fruits, vegetables, whole grains, tea, coffee, chocolate, red wine, legumes and other foods (136, 137). Based on the chemical structure of their aglycones, they are classified as phenolic acids, flavonoids, polyphenolic amides, and other polyphenols (138, 139).

Phenolic acids constitutes of benzoic and cinnamic acid derivatives. They are non-flavanoid polyphenols. Examples are caffeic acid and chlorogenic acid. Flavonoids on the other have two aromatic rings bound together by three carbon atoms (138). Flavonoids consist of six subtypes named based on the type pf heterocycle they contain. These are anthocyanidins, flavones, flavanones, flavonols. Isoflavones and flavanols (cathechins and proanthocyanins). Flavanols may exist as monomeric (Catechins), dimeric (Theaflavins) or polymeric (proanthocyanidins). Catechins are found predominantly in green tea, chocolate, red wine and fruits such as apricot. Theaflavins and thearubigins are formed by the oxidation of monomeric flavanols in black tea during fermentation (138).

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Polyphenol amides have N-containing functional substituents. Examples are capsaicinoids found in chili peppers and avenanthrmides in oats. Other polyphenols apart from the phenolic acids, flavonoids and phenolic amides include resveratrols, ellagic acid (dimer of gallic acid), hydrolysable tannins (glucose esters of gallic acid and ellagic acid), rosemarinic acid. Despite their role in human health, polyphenols are also known to possess anti nutrition properties. They are known as major dietary inhibitors of iron absorption (140). Polyphenols inhibit iron absorption by binding and forming complexes with iron within the intestinal lumen, after their release from the meals (141). This activity of polyphenols is mainly attributed to galloyl groups in polyphenols(142).

In a pioneering study, Disler et al. (143) investigated the effect of tea on iron absorption among multiparous housewives with a mean age of 40 years. They measured iron absorption from a test meal consumed alongside tea or water. They found that tea consumption inhibited iron absorption from different iron solutions ($FeCl_3$ and $FeSO_4$) as well as from bread and a rice meal. The results were attributed to tannins in the tea. Brune et al. (142) also investigated the importance of different phenolic structures in iron absorption. They found that tannic acid inhibited iron absorption in a dose-dependent manner, and to a similar extent as gallic acid. However, their findings suggested no inhibitory effect of catechin on iron absorption. In another study, Hurrell et al. (141) determined the effect of different polypohenol-containing beverages on iron absorption from a bread meal. Unlike Brune et al. (142) their study suggested that all major types of food polyphenols including chlorogenic acid, flavonoids, gallic acid, are potential inhibitors of nonheme iron absorption. Inhibition was highest with black tea (up to 94 %), and least with the herbal tea, camomile (47 %). Black tea is known to contain 10 %

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flavonols, 25 % catechins, 20 % theaflavins and 45 % thearubigins (144). Tuntawiroon et al. (145) also investigated the dose-dependent inhibitory effects of food polyphenols on nonheme iron absorption using Yod Kratin, a popular Thailand vegetable high in polyphenols (29.2 mg tannic acid equivalents per gram). They found that 20g of this vegetable (common serving size) could reduce iron absorption by approximately 90 % due to the high galloyl group content which is known to bind iron and inhibit its absorption. It has been shown that the inhibitory effect of polyphenols occurs when they are consumed together with the meal and that tea may inhibit iron absorption by forming complexes with the iron (146).

Calcium

Calcium is considered an essential element in human nutrition because it must be supplied to the body through the diet. The adult body contains approximately 1 kg of calcium which is mostly found in the skeleton where it provides skeletal support (147). In the United States, dairy products supply over 70 % of calcium intake (14). The inhibitory effect of calcium is has been well investigated in both human and animal studies. Deehr et al. (148) investigated the effect of different sources of calcium on iron absorption among 19 postmenopausal women. They found mean body retention of 8.3 % from the placebo. In the other treatments containing calcium from different sources, absorption was lower. Cook et al. (149) investigated the effect of three widely used calcium supplements (calcium carbonate, calcium citrate and calcium phosphate) on the absorption of nonheme food iron and also on the absorption of ferrous sulfate taken without food in adults with results supporting an inhibitory effect of calcium especially from an inhibiting meal.

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Calcium is unique among the dietary factors that influence iron absorption in that it is the only one with inhibitory effect on both heme and nonheme iron absorption. The nature of the inhibition effect of phytate has been described as flat inverse S-shaped, with no inhibition at <40 mg calcium in the meal and no further inhibition when calcium content exceeds 300 mg (150). This is based on a study in which Hallberg et al. (151) investigated the effect of different amounts of calcium on iron absorption. Iron absorption in this study was measured when a radiolabeled wheat roll contained no or known amounts of calcium added as calcium chloride either before or after baking. However, there is still uncertainty about how calcium interferes with iron absorption. Lynch (14) has suggested that the inhibitory effect of calcium on iron absorption may be explained by an interaction between calcium and food components that affect iron bioavailability or an effect of calcium on luminal surface receptors involved in iron uptake. However, Hallberg et al. (151) have suggested that the effect of calcium may be at the mucosal level, at a process common to both heme and nonheme iron.

Estimating Iron Bioavailability

There are many different approaches to assessing iron bioavailability. Based on its definition, bioavailability comprises of the digestibility (solubility), absorbability (absorption) of the iron and the utilization or its incorporation into a functional compartment or system (152).

Chemical balance method

The chemical balance method is an indirect measure of the iron retained in the body after ingestion. Apparent iron absorption is calculated from this method as the difference between iron intake and the fecal iron. This may be corrected by subtracting

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the urinary iron to estimate iron retention. Essentially since the urinary iron is minute, the retention iron and the apparent absorption are similar (152). This method has however been described as time consuming, insensitive and lacking precision owing to challenges such as incomplete fecal collection (9).

Solubility/dialyzability

The solubility approach to assessing iron bioavailability deals with only nonheme iron since heme iron is directly absorbed and enters the enterocyte as heme. In in vitro solubility measurement, the food sample is first treated with HCl to adjust the pH to two, after which it is digested with pepsin. After the pepsin digestion, the pH is adjusted to six and the sample is digested again with pancreatin. The soluble iron that is released is then measured in the supernatant after centrifugation (153). Iron dialyzability is a modification of the iron solubility method. This method involves the fractionation of the sample using a dialysis bag. The dialysis bag contains a base such as $NaHCO_3$ or PIPES buffer that gradually increases the pH to the level suitable for the action of the intestinal enzymes. This method measures dialyzable iron (the soluble iron with less molecular weight than the cut off of the dialysis tube) (152, 154-156). Iron solubility methods have the limitation that they only measure a part of the bioavailability concept, not taking into account the absorption and utilization components. Also dialyzability may not measure iron compound with higher molecular weight such as ferritin, although they may be absorbed in humans.

Caco-2 cell model

Caco-2 cells are human adenocarcinoma cells used widely in iron absorption studies. In the cell culture model, cells grown in a cell culture media at 37°C and CO_2

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concentration of 5 % are used in an iron uptake experiment. The test meal is digested in vitro as is done in iron solubility. The cells are then treated with the digested test meal to measure iron uptake from the digest by the cells (153). Despite minor discrepancies in the magnitude of the effects of dietary factors on iron absorption, the Caco-2 cells model has been demonstrated to correlation well with human absorption study data (153, 157). In one of such studies, Yun et al. (171) measured iron absorption using the Caco-2 cell model and compared the results to published studies on iron absorption measured in humans. They measured iron absorption from the meals when they contained ascorbic acid and tannic acid and also measured iron absorption when the foods these dietary factors were not added to the meal. They found a strong positive correlation between absorption ratio measured using the Caco-2 cell model and that determine in the human studies using extrinsic tagging ($r = 0.986$, $P < 0.001$). Although there are limitations to this model, it remains the best cell model for assessing iron bioavailability.

Hemoglobin repletion method

The rat hemoglobin repletion method is a standard method used by the Association of Official Analytical Chemist (AOAC) (158). It is usually used to determine the relative bioavailability value of a test iron compound in comparison with ferrous sulfate. In this method, male rats are kept on an iron depleted diet for a period of time and then fed an iron replete diet (containing the iron compound to be tested) for a period of time to improve their iron status (159, 160). Rats are usually housed individually in wire bottom stainless steel cages. They are kept under a 12-hour light-dark cycle. Food and deionized water are provided ad libitum over the entire study period (161). Blood collection before and after repletion is done using various methods including tail incision,

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orbital socket blood draw and cardiac puncture. In analyzing the data, changes in hemoglobin or hemoglobin iron during the repletion period is plotted against either iron intake or the iron level of the diet. In the slope ratio analysis, a single multiple linear regression model is constructed using the control (the no iron diet group) as the blank. This is done using the common intercept model. The slope for the test and the ferrous sulfate treatment are determined, and compared. The mean of the blank is compared to the intercept to determine if they are significantly different (159, 160). The hemoglobin repletion method is also used in piglets, however the use in rats is most common (162).

Isotopic methods

The isotopic methods are the most common methods for assessing iron bioavailability. The most accurate method is intrinsic labeling. In this method, the food is biosynthetically labeled with a radioisotope of iron. Despite its reliability, preparing the labeled food in the intrinsic tagging method is a difficult task. Cook et al. (163) found that iron absorption could also be measured with extrinsic tagging in which the food is labeled with the iron isotope during the iron absorption measurement. When they compared iron absorption measured by the two different methods, the results were same. Since extrinsic tagging is less cumbersome, it therefore became more popular than the intrinsic tagging method. This method is based on the underlying principle that all the nonheme iron from a given meal form a common pool and are absorbed with equal efficiency, thus implying uniform labeling of all the nonheme iron from the different components of a meal by a stable or radioactive tracer (9). There are two common techniques for measuring iron bioavailability using the extrinsic tagging method. These are the whole body counting method and the red cell radioiron incorporation. In the

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whole body counting method, a radioisotope of iron is administered with the test meal or the diet. For this method, ^{59}Fe is used because ^{55}Fe the other radioisotope of iron does not emit gamma radiations. A count of the radioisotope is made shortly (usually one hour) after the ingestion of the radiolabeled meal or diet to serve as the baseline count. This count is assumed to be 100 % (152, 164). The counts are taken again at approximately 10 to 14 days. After the first count, there is a continual decrease in ^{59}Fe as a result of fecal excretion. After 10-14 days, it is said to have reached equilibrium. The retained ^{59}Fe is estimated as the ratio of the final count at any given time to the initial count, expressed as percentage. The percentage iron absorption is calculated by first plotting a curve of the retention vs time and then extrapolating the straight line portion of the curve to the zero time (T = 0).

In the red cell incorporation method, subjects are fed a test meal that is labeled with either radio (^{55}Fe or ^{59}Fe) or stable (^{57}Fe or ^{58}Fe) isotopes of iron after an overnight fast. After consuming the test meal, subjects are required to abstain from food (only water allowed for the next several hours). Blood samples are collected two weeks after the test meal consumption to measure radioactivity in the blood. The absorption is usually calculated in comparison to absorption from a reference meal. Generally, the assumption is that at least 80 % of the absorbed isotope is incorporated into the red blood cells in two weeks (164).

Area under the curve for serum iron

This method is commonly used in pharmaceutical companies who use one of two post absorption measurements namely area under the curve or maximal concentration after drug/nutrient administration. It is a good method for comparing the absorption of

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two to three compounds in the same subject. In the serum iron curve method, post absorptive serum iron curve is constructed by collecting baseline blood sample, followed by test meal/compound administration and then the collection of multiple blood samples thereafter at regular time intervals (152). Blood samples are analyzed for serum iron and the curve for serum iron over time is constructed. The area under the curve for each subject is used as a measure for iron bioavailability. This approach was validated by Conway et al. (165). They reported a strong positive correlation between absorption measured by AUC and erythrocyte incorporation. Apart from the AUC, Conway et al. (165) also estimated iron bioavailability using the maximum value for serum iron in the serum iron curve, and also used values at 180 and 210 minutes. Of these options, they found that the maximum iron recovery at maximum gave the highest correlation with the erythrocyte incorporation of iron isotope.

Algorithms

Several statistical models have been developed recently for estimating iron bioavailability from diets. These are necessary because in population studies, the isotopic and other clinical measurements of iron absorption are not feasible, but rather laborious and expensive. Monsen et al. (108, 166) developed a model to estimate the bioavailability of dietary iron. Their model was based on iron absorption from heme and nonheme iron sources. For heme iron sources, percentage absorption is assigned based on level of iron stores, with 0, 250, 500 or 1000 mg iron stores corresponding to 35, 28, 23 and 15 % absorption respectively. This was based on the fact that heme iron absorption is influenced mainly by body iron levels and not by dietary factors. For nonheme iron, estimation of absorption was based on the levels of two different enhancers, namely

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ascorbic acid and animal tissue in the meal. These enhancers were the ones well studied at the time of their model. Depending on the amount of ascorbic acid and animal tissue in the meal, nonheme iron absorption was classified as high (8 %), medium (5 %) or low (3 %). In estimating iron absorption using this model, the total, heme and nonheme iron levels as well as the ascorbic acid and the animal tissue levels must be known. To estimate iron absorption from a day's meal, the iron absorption is first estimated for each single meal and snack separately, after which the iron absorption values are tallied up. This model was limited in that it did not include the effect of dietary inhibitors. The model was modified by Tseng et al. (167) to further adjust for the effect of tea and phytic acid. Du et al, (8) also developed a new algorithm after finding out that the existing algorithms of that time did not accurately predict iron bioavailability. Their new model incorporated ascorbic acid, animal foods, fruits, vegetables, beans, rice and tea. In that same year, both Reddy et al. (26) and Hallberg et al. (140) developed new algorithms. Hallberg's model was based on iron absorption from a basal meal multiplied by the effect of different dietary factors. The dietary factors considered in their model were phytate, polyphenols, soy protein, calcium, eggs, ascorbic acid, meat, and alcohol. The basal meal was a wheat roll that contains no inhibitors or enhancers of iron absorption. Absorption from this wheat roll was adjusted to that from a reference dose of 40 %. The wheat roll is made from low extraction wheat flour which has been well fermented to remove all inositol phosphates as much as possible. The effect of the different dietary factors were based on equations developed from absorption measurement made from the basal meal with and without the dietary factor at different levels. Their model also considered interactions between different dietary factors. A major limitation of this algorithm

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however was its complexity. Also their use of a wheat roll without any dietary inhibitors in measuring absorption was not realistic. Reddy's algorithm was developed using data from iron absorptions studies in which iron absorption was measured from 25 different meals among 86 subjects. Absorption values were adjusted to a ferritin level of 30 µg/L prior to the development of the model. They developed the model using multiple linear regression. After excluding polyphenols, calcium and nonheme iron intake due to lack of statistical significance in the model, the ascorbic acid, animal tissue and phytic acid remained as the significant predictors of nonheme iron bioavailability from the meals. Their data suggested that these dietary factors accounted for only about 16 % of variability in dietary iron bioavailability. Other authors have also developed other algorithms since then (12, 168, 169). The algorithm by Conway et al. (168) was based on food group consumption. They used published data in which iron absorption was measured from single meals labeled with radio-isotopes of iron. They adjusted iron absorption from these studies to correspond to 40 % absorption from a reference dose of 3 mg iron. This adjustment corresponds to iron absorption among subjects with borderline iron deficiency. Their model included the following food groups: animal tissue, high vitamin C fruit/juice, beans and lentils, whole grain cereals, tea, dairy, cheese, eggs, nuts and soya. Their algorithm explained 22 % of the variability in nonheme iron absorption. The algorithm developed by Rickard et al. (170) estimated available iron from the meal adjusting for the effect of ascorbic acid, phytate, meat, fish and poultry, calcium and polyphenols from tea. They also included nonheme iron intake as an inhibitor in their model considering that high intake of nonheme iron leads to a lower percentage nonheme iron absorption in the gut. They used data on iron absorption

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measured using the serum iron curve for a total of 228 test meals to develop this algorithm using selected non-linear functions. To estimate nonheme iron absorption using their model, a constant is first multiplied by the effect of each of two enhancers (ascorbic acid and animal tissue). The result is then divided by the effect of each inhibitor (phytate, polyphenol, calcium and nonheme iron). Their model explained 45 % of the variability in iron availability. When they used their model as well as those by Hallberg et al. (140) and Reddy et al. (26) to predict iron availability form an independent dataset, their algorithm gave values within reasonable range (5-35 %) while those by the two other models included predictions above 100 %. However, like other existing models, theirs was also developed from single meal studies data (165). The most recent algorithms were developed by Armah et al. (25) and Colling et al. (12). The model of Armah et al. (25) was based on published data from complete diet studies which investigated the effect of different dietary factors on iron absorption from a complete diet. The factors were meat, calcium, tea and ascorbic acid. In each of the four studies, the effect of the dietary factor was measured when subjects consumed typical, high or low levels of the factor in a five day diet. Iron absorption was measured using radioisotopes of iron (^{55}Fe and ^{59}Fe). Using a mixed effect model, they developed an equation that estimates the percentage of nonheme iron absorption from vitamin C, meat, calcium, tea (in cups as black tea equivalents), phytate, nonheme iron and also serum ferritin. This model was validated using both complete and single meal data from published studies with R-squared values of 84 and 57 % for complete diet and single meal data respectively. Their model suggested that serum ferritin was the most important factor predicting iron absorption among all the explanatory variables included in their model, while the contribution of

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dietary factors was small. Collings et al. (12) also recently developed a regression model that estimates nonheme iron absorption using serum ferritin concentration and the presence or absence of a dietary modifier. Their model was based on data from 5 published complete diet studies including a total of 58 subjects. Based on this model, they estimated the level of nonheme iron absorption for people with different ferritin levels. Their predicitions suggested that nonheme iron absorption could range from 1.8 to 23 % among individuals with ferritin 6-100 µg/L, depending on the nature of their diet. Their model suggested that the effect of dietary factors is more pronounced when iron status is poor (low ferritin) and less pronounced among individual with high iron status.

Performance of Existing Algorithms in Predicting Iron Absorption from Diets

Various authors have compared the different algorithms for their ability to predict iron absorption from the diet. In one such studies, Beard et al. (171) predicted iron absorption from the diets of Filipino nuns using six published algorithms (8, 26, 140, 166, 167, 169). Absorption values estimated using the different algorithms were lower than expected even when they estimated absorption for only subjects with ferritin less than 20 µg/L. Hunt (24) assessed the performance of six different published algorithms (8, 26, 140, 166, 167, 169) in predicting nonheme iron bioavailability from complete diets. She normalized the nonheme iron absorption values (both the observed and predicted values) to a ferritin level of 23 µg/L. While the Monsen algorithm (108, 166) predicted nonheme iron absorption from the high bioavailability diets well, the algorithms by Reddy (26) and Hallberg (140) better predicted the low bioavailability diets and overestimated absorption for high bioavailability diets. While the algorithms by Tseng (167) and Bhargava (169) underestimated nonheme iron absorption from the different diets, the remaining

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algorithms mostly overestimated. The best prediction of nonheme iron absorption according to the author was by the Hallberg model. In another study to investigate the role of low bioavailability diets in the development of iron deficiency among Moroccan children, Zimmermann et al. (11) estimated the percentage iron absorption from the diets of children 6-10 years old. They used the algorithms developed by Reddy et al. (26) and Tseng et al. (167) to estimate nonheme iron absorption setting heme iron absorption at 23, 28 and 35 % for high, medium and low iron stores respectively. The authors reported that the algorithm by Reddy et al. (26) gave a better prediction despite the fact that it was developed based on western type diet. Based on their estimation, iron absorption among the subjects was 0.22 mg/day, while the two algorithms predicted 0.21 mg/day and 0.55 mg/day for the Reddy (26) and Tseng (167) algorithms respectively. Since these comparisons of algorithms were published further new algorithms have been developed by Armah et al. (25) and Colling et al. (12) which have already been described above. Armah et al. (25) validated their model using published (independent) data, the resulting R-squared for single meal and complete diet data were 57 and 84 % respectively. The authors reported that their model overestimated nonheme iron absorption by 1.87 % which they attributed in part to the lack of complete data for the validation, citing the use of group means instead of individual dietary intake and serum ferritin values.

Single vs. Complete Diet Absorption Studies

Many of the existing studies on the effect of dietary factors on iron absorption were performed using single meals. In single meal studies, iron absorption is measured from a single test meal that is labeled with an isotope of iron. Alternatively, iron absorption from a whole diet can be measured. The whole diet refers to multiple meals

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such as all foods consumed in a day or number of days. In such cases, each meal component of the diet is labeled with the isotope of iron for iron absorption measurement. Studies have shown that when iron absorption is measured from the whole diet, the effect of dietary factors is less pronounced compared to what is observed in single meal absorption studies. In a study to compare iron absorption from single and complete diet studies, Cook et al. (27) measured iron absorption from both single meals and a two week diet among 45 subjects. Subject consumed either self-selected, enhancing or inhibiting diet for two weeks (n = 15 per diet). Iron absorption was measured from the two week diet as well as a single meal reflecting their respective diets (self-selected, inhibiting or enhancing meal). The outcome of their study showed that single meal absorption measurement give exaggerated estimates of the effect of dietary factors on nonheme iron absorption. Among those consuming self-selected diet iron absorption were similar when measured from single meal and complete diets. However, among those consuming the enhancing diet, while the single meal absorption measurement was 11.1 %, two week diet absorption was 6.6 %. Also in the inhibiting group, while the single meal showed iron absorption of 2.5 %, the complete diet absorption was 3.4 % suggesting the effect of the inhibitors was less than seen in the single meal absorption measurement. In another study among women with low iron stores, Hunt et al. (172) have shown that the effect of ascorbic acid on iron absorption from the diet is less than what is seen in single meal studies. This is also supported by observation made by Cook et al. (173) who assessed the effect of ascorbic acid on nonheme iron absorption from a complete diet. While the reason for the discrepancy between single and complete diet absorption values is not well

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understood a possible explanation for this observation in short-term studies is the residual effect of previously consumed meal components.

Conclusion

This review has covered iron metabolism, its function and the assessment and consequences of iron deficiency as well as strategies to address iron deficiency in populations. One key factor that contributes to iron deficiency in developing countries is iron bioavailability. We have discussed iron bioavailability and its assessment, as well as the dietary factors that influence the bioavailability of dietary iron. Many of these factors show a dose dependent relationship in their effect on iron bioavailability. Among the enhancers, the effect of ascorbic acid is more prominent and is able to counteract the inhibitory effects of both polyphenols and phytic acid. The enhancing effect of animal tissue is rather moderate, however with the associated heme iron, it contributes to total available iron, making it equally important in improving the intake of available dietary iron. It is also clear from this review that when measured from the whole diet, the effects of dietary factors are dampened compared to single meal absorption measurement. While there are suggested reasons, including adaptation and the residual effects of previous meals, data explaining the mechanisms are scanty, warranting future studies.

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CHAPTER 3. A COMPLETE DIET BASED ALGORITHM FOR PREDICTING NONHEME IRON ABSORPTION IN ADULTS

Modified from a paper published in *Journal of Nutrition*[1].

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Abstract

Many algorithms have been developed in the past few decades to estimate nonheme iron absorption from the diet based on single meal absorption studies. Yet, single meal studies exaggerate the effect of diet and other factors on absorption. Here, we propose a new algorithm based on complete diets for estimating nonheme iron absorption. We used data from four complete diet studies each with 12-14 subjects, for a total of 53 subjects (19 males and 34 females) aged 19-38 y. In each study, each subject was observed over three one-week periods during which they consumed different diets. The diets were typical, high or low in meat, tea, calcium or vitamin C intakes. The total sample size was 159 (53 X 3) observations. We used multiple linear regression to quantify the effect of different factors on iron absorption. Serum ferritin was the most important factor in explaining differences in nonheme iron absorption, whereas the effect of dietary factors was small. When our algorithm was validated with single meal and complete diet data, the respective R^2 values were 0.57; P<0.001 and 0.84; P< 0.0001. Results also suggest that between-person variations explain a large proportion of the

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differences in nonheme iron absorption. The algorithm based on complete diets we propose is useful for predicting nonheme iron absorption from the diets of different populations.

Introduction

Iron deficiency anemia is a leading global problem mostly attributed to low intakes of dietary iron and also poor iron bioavailability. Efforts to explore the relationship among iron intake, bioavailability, absorption and status, have led to the development of several algorithms to predict iron absorption in the past few decades. These have been reviewed by Reddy (1) and also more recently a new algorithm was published by Rickard et al. (2). A major limitation of the published algorithms is that they were developed based on single meal absorption studies (2-4). It is well known that the effect of dietary factors on iron bioavailability is exaggerated in single meal studies (5). For example, when iron absorption was measured from a 5-d complete diet, the effect of meat, calcium and ascorbic acid were diminished (6-8). In a human study, Hunt and Roughead (9) reported a decrease in nonheme iron absorption among men consuming high bioavailability diets and an increase among those on low bioavailability diet after 10 wk of feeding suggesting that individuals adapt to the effect of dietary factors on iron absorption. Although vitamin C is known to increase iron absorption from single meal feeding studies, 2 g/d vitamin C supplementation for 16 wk had no effect on iron stores (10). The above studies suggest that the effect of dietary factors on iron absorption is dampened with longer periods of consumption, and that there is the possibility that adaptation may occur over prolonged exposure to iron inhibitors or enhancers. Hence, the

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lack of agreement between predicted absorption from existing algorithms and iron absorption measured from whole diets and long-term studies is not unexpected (11, 12).

Predicting iron absorption from a complete or whole diet thus requires an algorithm that is developed from a complete diet. The complete diet here refers to a person's total daily intake estimated from one or more days. Studies have been conducted to examine the effect of different dietary factors on iron absorption from a complete diet consumed over a 5-d period (6-8) and unpublished data). Detailed nutrient intakes and absorption measurements have been published from these studies. In each of these studies, participants consumed three different diets, each during a period of five days. The diets included in the studies were a typical diet and two modified diets with low and high levels of the factor tested in the particular study. The four dietary factors that were included in three different levels in the four studies were tea, meat, vitamin C and calcium. By combining data from these studies we can approximately recreate the heterogeneity of typical diets consumed by various populations. The objective of this study was therefore to develop a new algorithm based on complete diet data from these studies, which will be useful in assessing iron absorption in populations.

Methods

Data Description. We used data from four different studies that were designed to measure nonheme iron absorption from a 5-d complete diet using extrinsic radio iron labeling technique (6-8) and unpublished data). Each study included 12-14 subjects (19 males and 34 females) aged 19-38 y, with a total of 53 subjects. All subjects were healthy with no history of disorders known to affect iron absorption. The studies were designed to determine the effects of ascorbic acid, meat, tea and calcium on nonheme iron

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absorption from a complete diet. Each subject consumed diets with low, typical and high levels of the dietary factors being studied (ascorbic acid, tea, meat or calcium). Absorption of nonheme iron was measured for each dietary period using extrinsic radiolabeling technique by labeling each of the three main meals of the day for 5 d and snacking in between meals was not allowed, except for the calcium study where preliminary data indicated that most subjects consumed two main meals, thus only the two main meals were labeled (7). Even with the snack consumption, the effect would be very small on iron absorption since they were not taken together with the meals. Each study reported mean daily nutrient intake during the labeling period, iron status (baseline serum ferritin) and percent iron absorption. Dietary records were kept by subjects and nutrient intakes were determined using the NUTRITIONIST IV program (N-squared Computing, First Data Bank Division, Hearst Corporation, San Bruno, CA). Phytic acid was not reported in those studies, however, we determined the phytic acid content using the Nutrition Data System for Research (University of Minnesota Nutrition Coordinating Center) since we had access to the complete dietary data. Tea consumption was estimated using black tea equivalents as follows: 2 cups (480mL) of iced tea and 1.5 cups (360mL) of herbal tea or coffee were coded as 1 cup (240mL) of black tea (13). One cup (240ml) of black tea equals I tea bag (1.9g of black tea). Other details about data collection procedures have been described elsewhere (8). Data collection procedure for the tea study (unpublished data) was similar to those described above and was approved by the Human Subjects Committee at the Kansas University Medical Center. Since each respondent had three data points for high, low and typical intake of the specified factor, the total number of observations in the combined data was 159.

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Data Analysis. We used R software version 2.12.2 to carry out the data analysis. The factors we considered in the analysis were serum ferritin, calcium, phytic acid, ascorbic acid, tea, nonheme iron, and meat, fish and poultry (MFP). We transformed all variables using a log transformation to better approximate normality. To develop the algorithm, we fitted a linear multiple regression model using the above factors to predict percent iron absorption. We included a person-level random effect in the model because each individual contributed three different observations and this induces intra-class correlation. We obtained maximum likelihood estimates of fixed effects and variance components (the latter based on a restricted likelihood function) and best linear unbiased predictors of the person-level effects. *P*-values were considered significant if $P \leq 0.05$, and weakly significant if $0.05 < P \leq 0.1$. We reported the median (10th, 90th percentiles) for the dietary factors and the geometric mean (10th, 90th percentile) for serum ferritin and absorption to avoid the influence of extreme values since the data are not normally distributed.

Validation of algorithm with human iron absorption data. We assessed the validity of the algorithm by estimating absorption using dietary intake and serum ferritin data from different published studies (both single meals and complete diets), and comparing results with reported absorption values. The single meal study included mean values for nutrient intakes and absorption from 19 different meals (4) which was designed to measure nonheme iron absorption from single meals fed after an overnight fast. Similarly we validated our algorithm separately using data from published complete diet studies (9, 14-17). The data from these studies were the mean values for dietary intakes, initial serum ferritin concentration, and unadjusted absorption for different arms

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of each study (n=23). In cases where the mean ferritin values were not given for the different arms, we used the mean for the whole group for each arm. In some of the data from the study by Tetens et al. (15) the quantity of tea consumed was not reported. We therefore assumed one cup if tea was included in the meal and validation was done with and without those data points.

Results

Serum ferritin concentration ranged from 5 to 162 µg/L (n=53), with geometric mean of 41.2 µg/L when all studies (6-8) and unpublished data) were combined. Since each of the 53 subjects consumed three different diets in the various studies, the dietary intakes presented in **Table 1** gives the median (10th, 90th percentiles) of the different factors by diet type and for all diets put together.

Results from the mixed effects model (**Table 2**) suggest that between-person differences explain a large proportion of the variation in nonheme iron absorption. This is reflected by the equal variances for the residual and the grouping variable (ID) for the random effect. Although the dietary factors had minimal contribution in explaining variations in absorption, their inhibitory or enhancing effect was as expected. However, serum ferritin had a highly significant effect on absorption ($P < 0.0001$), explaining approximately 35 % of the variance in absorption as shown under the partial R^2 column. The residual plot (**Figure 1A**) shows no pattern when we plot standardized residual against the fitted values and there is good correlation between observed and fitted absorption (**Figure 1B**) suggesting that the model fits the data well. The fitted regression model is:

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$$Ln\ Absorption\ (\%) = 6.294 - 0.709\ ln\ (SF) + 0.119\ ln\ (C) + 0.006\ ln\ (MFP+0.1) - 0.055\ ln\ (T+0.1) - 0.247\ ln\ (P) - 0.137\ ln\ (Ca) - 0.083\ ln\ (NH)$$

where SF is Serum ferritin (µg/L), C is vitamin C (mg), MFP is meat, fish and poultry (g), T is tea (number of cups), P is Phytate (mg), Ca is calcium (mg) and NH is nonheme iron (mg).

Validation of model for predicting nonheme iron absorption. We observed a significant positive correlation (R^2=0.57; $P < 0.001$; **Figure 2A**) when we fitted the proposed model to predict nonheme iron absorption from the single meal study that reported mean values for 19 meals (4). Similarly, we found a significant correlation (R^2=0.84, $P < 0.0001$; **Figure 2B**) between predicted absorption and measured absorption from complete diets data. The R^2 did not change (R^2 = 0.84) when we excluded the data points from the Tetens et al. (15) study that did not have the quantity of tea consumed.

Discussion

The magnitude of the global problem of anemia makes it important to determine means of estimating the percentage of iron absorbed, particularly from nonheme sources, since they provide the main sources of iron in many developing countries. Accurately estimating iron absorption is also essential to set dietary recommendations. Since iron absorption from single meals exaggerates the effect of dietary factors (5, 9, 12), there is concern about the use of algorithms based on single meals to estimate iron absorption from a whole diet. By overestimating absorption, we risk underestimating the prevalence of inadequate iron intakes. This is one reason why development of an algorithm based on complete diets that can accurately predict iron absorption is critical.

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The algorithm we propose was developed from complete diet data sets that include a wide range of intake of the dietary factors known to affect nonheme iron absorption, and therefore should reflect the consumption pattern of many populations. Maximum intakes for most dietary factors were extremely high due to the designs of the studies from which the data were obtained. For example, the maximum intake of vitamin C was 584 mg which far exceeds the usual intake in most populations. Similarly the very low minimum intakes make the algorithm useful for populations with extremely low intakes of these dietary factors. For instance, the model can be used to estimate iron absorption among vegetarians and among people who do not consume tea or coffee habitually. Besides, tea estimation in our study as number of cups (in black tea equivalents) is much easier than estimating polyphenols content since inaccuracies exist in estimating different types of polyphenols.

Most of the existing algorithms require estimating iron absorption from each meal separately to obtain total daily iron absorption (3, 4, 18). When wide ranges of total daily nutrient intakes are used with singles meal algorithms the result may be inaccurate. For instance, when we predicted nonheme iron absorption from the published complete diet data sets (9, 14-17) using the Reddy et al. (4) and the Hallberg and Hulthén (3) algorithms, some of the predicted absorption values for both algorithms were above 100 % due to the wide ranges of intakes used. A similar observation was made by Rickard et al. (2) and Beard et al. (12). However, with our proposed algorithm, total daily nutrient intakes may be used without the need to calculate bioavailability for individual meals. In addition, this algorithm incorporates iron status (serum ferritin) in the model unlike

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previous algorithms. Including serum ferritin in the model avoids any errors that can arise when adjusting for serum ferritin using another equation.

Of the different factors considered in this algorithm, serum ferritin had the strongest effect in the model. It was not surprising to see that the effect of dietary factors was minimal, because even with a single meal study in which dietary factors were accurately measured in the laboratory, only 16 % of variation in absorption was explained by dietary factors after adjusting for serum ferritin (4) . Cook et al. (5) have also shown that in the whole diet the effect of dietary factors is dampened, supporting our findings. Among the dietary factors, only phytic acid was weakly significant in the model (P = 0.09).While we expect minimal effect of these dietary factors as indicated by the literature, it is also possible that phytic acid was weakly significant because it is mostly a component of the iron containing meal and can therefore chelate nonheme iron to reduce its bioavailability. Including higher forms of inositol phosphates (hexa and penta inositols) could give a better model, however these are rarely found in food composition databases compared to total phytate. Since the aim of our study was to provide more convenient model for use in population studies rather than one that requires laboratory analysis of foods, total phytate content was used in developing the model.

Although polyphenols have strong inhibitory effect on iron absorption, they didn't show much effect on our model. While it would have been more appropriate to use polyphenol content instead of black tea equivalents that would make the model more demanding to use because foods/beverages have different forms of polyphenols. Besides there are different methods for measuring polyphenols and the results differ from method to method. Studies have reported polyphenols content as gallic acid or catechin

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equivalents. To avoid difficulties related to type and method of polyphenols analysis we included in the manuscript conversion factor for different beverages to black tea equivalents. The conversion factors take into account the polyphenol contents of the different beverages and are easy to use in the population studies. Based on previous studies showing the effect of different types of beverages on iron absorption (13), it is reasonable to estimate polyphenols using black tea equivalents rather than absolute amounts. One cup of black tea contains 200-250 mg catechin equivalents.

Other factors such as tea, calcium and ascorbic acid may not necessarily be consumed together with the meals. For example Morck et al. (19) and South et al. (20) have shown that tea had to be consumed with the meal to inhibit iron absorption. Also dairy products as sources of calcium may be consumed separate from the meal, thus minimizing their effect on nonheme iron absorption (21). The results of this study support the findings by Reddy et al. (6) that the contribution of heme iron by MFP is more important in improving iron status than in promoting nonheme iron absorption.

When we validated our proposed algorithm with complete diet and single meal studies data the R^2 values were 0.84 and 0.57, respectively. These results suggest that this algorithm can be used to predict absorption of nonheme iron for both complete and single meal studies. The fact that the algorithm predicted better for the complete diet than for the single meal data sets was not surprising since it was developed from complete diet datasets. To compare our algorithm with the three most recently developed algorithms (2-4), we predicted nonheme iron absorption using complete diet data (9, 14-17) and each of the different algorithms. These algorithms gave lower R^2 values (0.64, 0.69 and 0.72 for the Rickard et al. (2), Reddy et al. (4), and the Hallberg and Hulthén (3) algorithms

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respectively). Further, some of the predicted absorption values arising from the Reddy et al. (4) and Hallberg and Hulthén (3) algorithms exceeded 100 % as mentioned above.

Despite the fact that most of the dietary factors did not contribute significantly in explaining variation in absorption, at least based on the data used for this algorithm, we included them all in the model to adjust for any difference in their intakes among subjects. Our study also shows that between-person differences contribute to the variations in iron absorption. In a recent review by Hurrell and Egli (22), it was indicated that besides iron status, other host factors such as inflammation and obesity may play a significant role, which supports our observation of high between-person variability in absorption. Further studies are recommended to probe this area of research.

One limitation of this algorithm is that all the four data sets used were obtained in the United States. However, the diets included wide ranges of intakes with dietary modification which makes the algorithm applicable to other countries. In conclusion, we have developed a user friendly model for estimating nonheme iron absorption from the complete diet. This model can predict nonheme iron absorption with high accuracy and will be useful in estimating iron absorption among populations. To estimate nonheme iron absorption using our proposed algorithm we recommend the use of mean daily intake of nutrients estimated from three or more days' dietary records, and serum ferritin concentration preferably individual subject values.

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Table 1 Mean daily intakes for dietary factors, serum ferritin concentration and nonheme iron absorption by diet type[1]

Factor/Nutrient	Typical[2]	High[2,3]	Low[2,4]	Total
Phytate, *g*	0.61 (0.34, 0.86)	0.53 (0.30, 1.02)	0.61 (0.35, 0.98)	0.57 (0.33, 0.96)
Nonheme iron, *mg*	10.3 (6.9, 19.3)	9.9 (6.5, 18.5)	10.7 (6.4, 17.2)	10.3 (6.5, 18.6)
Calcium, *g*	0.81 (0.40, 1.23)	0.88 (0.39, 1.40)	0.65 (0.22, 1.26)	0.77 (0.31, 1.35)
Ascorbic acid, *mg*	85 (32, 196)	78 (27, 256)	54 (28, 135)	73 (28, 236)
MFP[5], *g*	94 (39, 228)	108 (43, 242)	82 (0, 162)	91 (11, 215)
Tea[6], *cups*	0.0 (0.0, 0.6)	0.1 (0.0, 3.0)	0.0 (0.0, 0.7)	0.0 (0.0, 1.1)
Serum ferritin[7], *µg/L*	41 [17, 86]	41 [17, 86]	41 [17, 86]	41 [17, 86]
Absorption[7], *%*	4.3 [1.6, 12.4]	5.3 [1.8, 20.5]	5.7 [1.9, 14.5]	5.0 [1.7, 16.9]

[1]Values are from four different studies (6-8 and unpublished data)
[2]Reported values are median (10th, 90th percentile), n = 53
[3]Diet was high in one of four dietary factors (meat, ascorbic acid, calcium or tea)
[4]Diet was low in one of four dietary factors (meat, ascorbic acid, calcium or tea)
[5]MFP: Meat, fish and poultry
[6]1 cup (240mL) of black tea= 2 cups of iced tea or 1.5 cups of herbal tea or coffee
[7]Reported values are geometric mean [10th, 90th percentile]

Table 2 Summary for mixed model for predicting nonheme iron absorption (%).

Effect	Group name/Factor	Coefficient	SE	t-value	*P*-value	Partial R^2 (%)
Random effects	ID[a] (Intercept)	-	0.07	-	-	
	Residual	-	0.07	-	-	
Fixed effects[b]	Intercept	6.294	1.04	6.06	<0.0001	
	Ferritin, *µg/L*	-0.709	0.10	-6.88	<0.0001	35.30
	Ascorbic acid, *mg*	0.119	0.07	1.62	0.11	0.79
	MFP, *g*	0.006	0.02	0.28	0.78	0.03
	Tea, *cups*	-0.055	0.04	-1.28	0.20	0.09
	Phytate, *mg*	-0.247	0.15	-1.70	0.09	0.84
	Calcium, *mg*	-0.137	0.09	-1.49	0.14	0.01
	Nonheme iron, *mg*	-0.083	0.17	-0.50	0.62	0.81

[a] Stands for subjects' ID
[b]All fixed effect variables were log transformed using natural log

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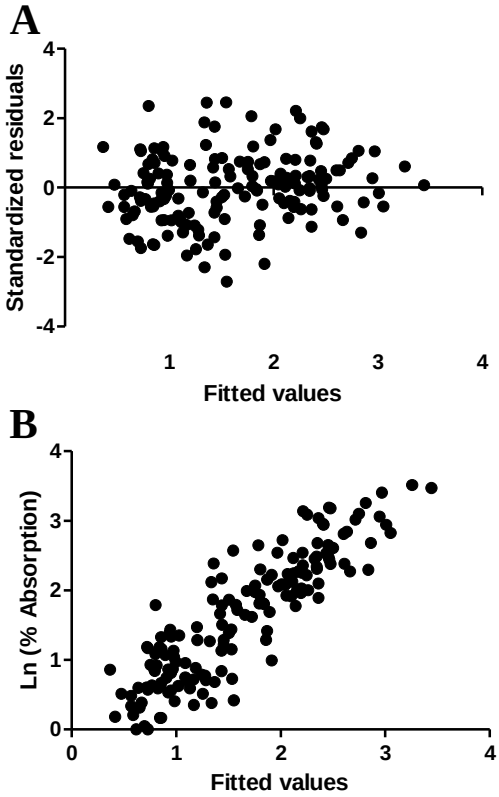

Figure 1 Diagnostic plots for the complete diet based nonheme iron absorption model. Residual plot of standardized residuals vs fitted values (A), and a plot of observed percent absorption in the natural log against the fitted values (B).

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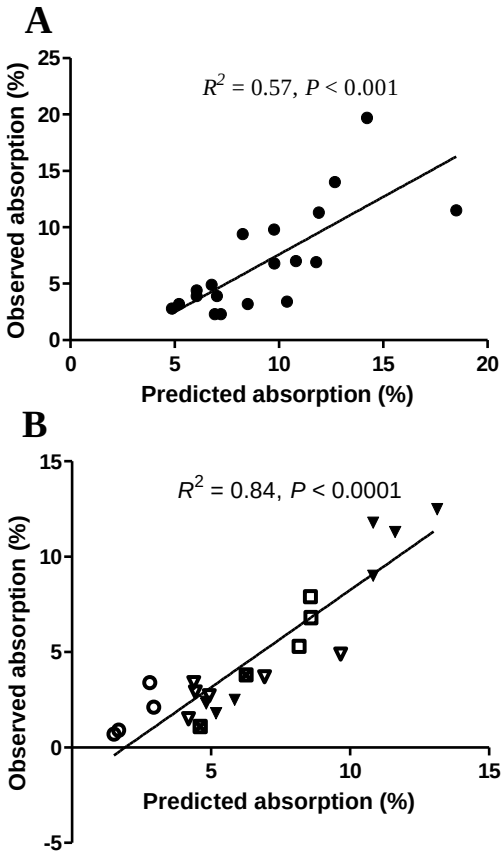

Figure 2 Observed vs. predicted absorption for single meal (A) and complete diet (B) data using the complete diet based iron absorption algorithm. When we used our algorithm to predict absorption from published data, the R^2 values were 0.57 (n=19; P< 0.001) and 0.84 (n=23; P<0.0001) for single meal and complete diet data, respectively. For B, data were from five different studies: ○(9), ▼ (14), ▽ (15), □ (16), ⊠ (17). The predicted values were estimated using reported mean dietary intakes and serum ferritin concentration for different arms of each study. In cases where mean ferritin for the different arms were not provided in the study, the overall mean was used for each arm.

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CHAPTER 4. ESTIMATING IRON BIOAVAILABILITY FROM THE US DIET

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Abstract

Iron bioavailability from the US diet has been estimated to be 18 %. This value was derived from a single human absorption study. New data arising from recent studies, however, suggest that it may be time to revisit this bioavailability estimate. In this study, we estimated iron bioavailability from the US diet using our recently reported complete-diet based algorithm. We used dietary intake and biomarker information from the National Health and Nutrition Examination Survey (NHANES, 2001-2002), MyPyramid Equivalents Database (MPED), and the Food and Nutrient Database for Dietary Studies (FNDDS). The survey package in R was used for estimating means, percentiles and their confidence intervals taking into account the strata, primary sampling units (PSU) and the appropriate survey weight. We implemented two different approaches to estimate total absorption. In the first approach, we used all survey participants but adjusted the geometric mean of nonheme iron absorption to 15 μg/L ferritin to mimic values of individuals with no iron stores; in the second approach, absorption was estimated only for non-anemic subjects with no iron stores. In both approaches, we considered estimating bioavaiability for subjects with no iron store since that is conventionally done to estimate the upper ceiling of absorption. A total sample size of 6,631 was used based

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on availability of dietary and iron status biomarker data and C-reactive protein (CRP) concentration ≤ 6 mg/L. In the sample, mean ferritin, CRP and haemoglobin concentrations were 58.4 µg/L, 1.6 mg/L and 14.3 g/dL respectively. Mean intakes of vitamin C, calcium, tea (black tea equivalents), meat fish and poultry (MFP), nonheme iron and phytate among sample individuals were 94 mg, 920 mg, 0.3 cups, 128 g, 14.3 mg and 719 mg respectively. Nonheme iron absorption ranged from 1.9 % among men (19-50 years) to 7.3 % among children 3-8 years. The estimated total iron absorption was 15.49 % or 15.06 % depending on which approach was used to carry out the calculations. This study provides useful data for evaluating the current value of iron bioavailability from the US diet.

Introduction

Bioavailability has been defined as the fraction of the ingested nutrient that is absorbed and subsequently utilized for normal physiological functions (1). Iron bioavailability is of interest because poor absorption is considered to be a major contributing factor to the prevalence of iron deficiency worldwide. In the developing world alone, over 3.5 billion people are either iron deficient or have iron deficiency anemia (2). In the United States, the prevalence of iron deficiency was estimated to be 9 % among non-pregnant women 20-49 years old using the currently popular body iron model (3). When the relatively older ferritin model is used, however, the estimated prevalence of iron deficiency jumps to 16 % (3). Total iron absorption from the diet can be estimated by combining the fractional absorptions from nonheme and heme iron sources. While it is widely accepted that approximately 25 % of heme iron is absorbed (4), there is considerable variation in the proportion of nonheme iron that is absorbed, because it is influenced markedly by iron status and to some extent by dietary factors (5). Thus, estimates of nonheme iron bioavailability have differed

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greatly in the literature as a result of different combination of personal and dietary factors (6-12).

Hallberg et al. (13) estimated that total iron bioavailability for a typical Western-type diet may range from 14 –17 % among subjects who are border-line deficient in terms of iron (serum ferritin $\leq$ 15 µg/L). In their work, they considered bioavailability estimates for Swedish, French and US diets. The World Health Organization (WHO) has proposed a bioavailability of 15 % for Western-type diets that are high in fruit, vegetables, meat and fish, and of 12 % for a more typical Western diet with less intake of the above mention foods (14). In the US and Canada, the U.S. Institute of Medicine (IOM) and Health Canada have established that total iron bioavailability is 18 %. This estimate was based on data from a study in which absorption was measured among 15 subjects who consumed their typical diet for two weeks (15, 16). The current estimate of total absorption is based on nonheme iron absorption from the above study corrected to a ferritin concentration of 15 µg/L plus heme iron with absorption assumed to be 25 %.

There are several reasons why it is critical to accurately estimate the iron bioavailability in the diet of Americans. First, bioavailability is required for setting the Dietary Reference Intakes (DRIs). For example, in setting the current DRIs for iron, 18 % bioavailability was used for most age groups, except for pregnant women and children under 1 year of age (17). If iron bioavailability is over-estimated or under-estimated, then the risk of iron inadequacy at different intake levels would be set to be too low or too high, respectively(5). Second, iron bioavailability must be known if we wish to estimate the prevalence of inadequate intake of iron in a population (that is, the proportion of individuals in a population whose iron intakes do not meet requirements). If the bioavailability of iron in the diet is over-estimated, this may

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result in underestimation of the prevalence of inadequate iron intake and vice versa. An estimate of bioavailability is also required to design and implement nutrition policies such as food fortification programs.

Iron bioavailability can be measured using stable or radioactive isotopes of iron (8, 18-21). However, these methods are not feasible in population studies because they are expensive and cumbersome. Thus, several algorithms have been developed for estimating iron bioavailability in the population (22-31) but their limitation is that they are based on single meal studies. As a result, estimates of bioavailability can be variable because dietary factors that affect iron absorption (including phytate, vitamin C, polyphenols, calcium and animal tissue) may vary across single-meal isotope studies and can affect estimation of bioavailability. Hunt (32) argued that this lack of repeatability among single-meal models to predict human absorption raises the need to develop algorithms that are based on complete-diets (32).

In this paper, our main objective is to estimate iron absorption from the US diet using a complete-diet algorithm that we recently proposed (33). Unlike previously published algorithms, this algorithm takes into account iron status in the population and was developed using complete diet data. The National Health and Nutrition Examination Survey (NHANES, 2001-2002)(34), My Pyramid Equivalents Database (MPED version 1.0) (35), and Food and Nutrient Database for Dietary Studies (FNDDS version 1.0) (36) provide nationally representative data with the information needed at the individual level to estimate iron absorption from the US diet using the algorithm we have proposed. The manuscript is organized as follows: In the methods, we describe the sources of data, inclusion criteria, and the procedures for the estimation of nutrients and dietary factors intake, especially for phytate and tea equivalents which were not reported in the NHANES data. We further explain the

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calculation of nonheme and total iron absorption. Our estimates of the dietary intakes and iron status biomarkers by demography as well as nonheme and total iron absorption are presented in the results section. Comparison of our results to existing literature and limitations of the study are discussed and a conclusion is offered at the end of the discussion section.

Methods

Data sources and general procedures. In this analysis we used NHANES data (2001/2002), MPED version 1, and FNDDS versions 1.0. These data were used because the more recent ones do not have information on iron status biomarkers for men. We used day one dietary intakes and serum ferritin values for NHANES 2001/2002 for estimating absorption. Only those individuals with complete information for the variables needed to estimate iron absorption were included in the analysis (n=6,631). Pregnant and lactating women as well as individuals with C-reactive protein (CRP) > 6 mg/L were excluded from the analyses.

Food and nutrient intakes estimation. We obtained individual-level daily nutrient intake of vitamin C, iron and calcium from the NHANES, and estimated nonheme iron intake to be 90 % of total iron intake (15). Since NHANES does not report phytate intake, we estimated phytate intakes using information about the phytate content of phytate-containing foods as reported by the International Zinc Nutrition Consultative Group (IZiNCG) (37). The IZiNCG report lists phytate content of different foods and food groups. Using these data we estimated the phytate content of the various food groups reported in the MPED equivalence data. These values were scaled appropriately in order to obtain the phytate content in mg/100 g of each food group. We merged the resulting data with the Individual Foods data from NHANES to estimate the phytate content of each food consumed by each study participant. The

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total daily phytate intake was the sum of the phytate amounts contributed by the different foods consumed by the individual during the day.

We estimated polyphenol intake as black tea equivalents (cups) since the polyphenol content in foods and beverages is not reported in NHANES. Tea, coffee and related beverages reported in the FNDDS were assigned black tea equivalents using the following conversion factors: 2 cups (480mL) of iced tea and 1.5 cups (360mL) of herbal tea or coffee were coded as 1 cup (240mL) of black tea (38). These values were used to estimate the total number of cups consumed by each subject.

Consumption of meat, fish and poultry (MFP) was estimated using the appropriate MPED data which provide daily intake of MFP for each subject in MyPyramid equivalents. The MFP equivalents were converted to grams using a conversion factor of 28.35g per ounce.

Nonheme iron absorption was estimated using the following equation (33):

$$Ln\ Absorption\ (\%) = 6.294 - 0.709\ ln\ (SF) + 0.119\ ln\ (C) + 0.006\ ln\ (MFP+0.1) - 0.055\ ln\ (T+0.1) - 0.247\ ln\ (P) - 0.137\ ln\ (Ca) - 0.083\ ln\ (NH),$$

where SF is Serum ferritin (µg/L), C is vitamin C intake (mg), MFP is meat, fish and poultry consumption (g), T is black tea equivalents (number of cups), P is estimated phytate (mg), Ca is calcium (mg) and NH is nonheme iron (mg).

Nonheme iron absorption was estimated at the individual level and geometric means and percentiles were estimated for different groups and subpopulations.

In estimating total iron absorption for the US population, we used two different approaches. In the first approach we adjusted the geometric mean of nonheme iron absorption to a serum ferritin concentration of 15 µg/L. In the second approach, we estimated iron absorption choosing only the subjects who were iron deficient (nonanemic but with ferritin value $\leq$ 15 µg/L). The procedure for the first

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approach was as follows: Nonheme iron absorption was adjusted for ferritin (15 µg/L) using the following equation which was developed based on the inverse relationship of ferritin and nonheme iron absorption (16).

Log [Adj Abs (%)] = log [Obs Abs (%)] + log [Obs Ferritin (µg/L)]

- log [15 (µg/L)]

where *Adj Abs* is adjusted nonheme absorption, *Obs Abs* is observed nonheme absorption (geometric mean), and *Obs Ferritin* is observed serum ferritin (geometric mean). To estimate total absorption, we used the following equation (15):

Tot Abs (%) = (Adj Nonheme Abs (%) X 0.9) + (Heme Abs X 0.1),

where *Tot Abs* is total absorption, *Adj Nonheme Abs* is the adjusted nonheme iron absorption and *Heme Abs* is heme iron absorption.

We used a conservative value of 25 % for heme iron absorption (4), and assumed that 90 % of dietary iron in the typical US diet is nonheme with 10 % as heme iron (15).

In the second approach, instead of adjusting the geometric mean of nonheme iron absorption to 15 µg/L, we estimated absorption for only a sub-sample of the subjects who have no iron stores but are not anemic (13, 15). The presence or absence of anemia was established using WHO cut-off values for hemoglobin concentration (39). To determine whether an individual had no iron stores, we used the threshold: serum ferritin $\leq$ 15 µg/L. Thus, we excluded anemic individuals as well as individuals with ferritin greater than 15 µg/L.

We used the R software version 12.2.2 for statistical analysis. We estimated percentiles, means and confidence intervals using the survey package in R taking into account the strata, primary sampling unit (PSU) and appropriate sampling weight. Student t-test (2-tailed) was used to compare means of nonheme iron absorption and

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dietary factor intakes among sub-populations. Statistical significance was set at $p<0.05$. Serum ferritin and nonheme iron absorption were log-transformed using the natural log to approximate normality. The transformed values were used for all statistical analyses/tests and final estimates were converted into original scale.

Results

Summaries by age and gender. In these analyses, mean daily intakes for vitamin C, calcium, phytate and MFP for all subjects were 94 mg, 920 mg, 719 mg and 128 g respectively (**Table 1**). Average daily polyphenol beverage intake for all subjects (both drinker and non-drinkers) in black tea equivalents, was 0.30 cups. Intake of most dietary components were highest among men (19-50 years) except for calcium and tea equivalents. Mean nonheme iron intake ranged from 11.8 mg (postmenopausal women) to 17.2 mg (men 19-50 years). Geometric mean of hemoglobin concentrations ranged from 13.1 g/dL in children 3-8 years to 15.6 g/dL in men 19-50 years, while mean CRP was highest among postmenopausal women (2.40 mg/L). The geometric mean of serum ferritin for all subjects was 58.4 µg/L. Serum ferritin concentrations were highest among men 19-50 years (132 µg/L), and least among 3-8 year old children (25 µg/L). Nonheme iron absorption was lowest among men 19-50 years (1.9 %). Various percentiles of the observed distribution of the different components and nonheme iron absorption are shown in **Table 2.**

Summaries by ethnicity. Further, demographic differences in consumption of the dietary components we consider and in ferritin concentrations are shown in **Table 3. Figure 1** compares nonheme iron absorption by ethnicity (A) and gender (B). Nonheme iron absorption was significantly lower for non-Hispanic Whites than for both non-Hispanic Blacks ($P < 0.0001$) and Mexican Americans ($P < 0.0001$). Non-Hispanic Whites had lower vitamin C and MFP intake and higher ferritin

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concentration, calcium, phytate and tea intakes than non-Hispanic Blacks ($P < 0.05$) (P-values not shown in the table). Similarly, non-Hispanic Whites had higher ferritin concentration and tea intake and lower vitamin C intake than Mexican Americans ($P < 0.05$). These differences may explain the variation in estimated iron absorption. When comparing males and females, we found that nonheme iron absorption was higher in females (5.55 %) than in males (2.56 %). This difference in absorption was significantly different from zero ($P < 0.0001$). Males had higher intake of all dietary components associated with iron absorption than females and all the differences were statistically significant ($P < 0.05$) except in the case of intake of black tea equivalents ($P = 0.45$). Females also had a lower serum ferritin concentration than males (35.5 µg/L and 92.2 µg/L for females and males respectively) ($P < 0.0001$).

Total iron bioavailability estimation. After correcting nonheme iron absorption to a serum ferritin of 15 µg/L and adding fractional absorption from heme iron, the percentage total absorption was 15.49 % (n= 6,631). When we estimated absorption using the second approach (only subjects who were nonanemic but lacked iron stores, n = 678), the total iron absorption was 15.06 %.

Discussion

Despite global efforts to improve iron nutrition in at-risk populations, iron deficiency anemia remains a challenge (40). In the US, among most at-risk populations, the prevalence of iron deficiency is high even at present (41, 42) even though the US diet is high in sources of heme iron and wheat and other grain flour is fortified with highly available iron. One possible explanation for this persistent deficiency is that the bioavailability of iron in the diet of Americans has been over-estimated; as a consequence, the prevalence of inadequate intakes may have been under-estimated. In this study, we revisit the problem of estimating iron absorption

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from dietary intake and iron status information. To do so, we use data from participants in NHANES, a nationwide food consumption and health survey.

From the survey, we estimated mean daily calcium and vitamin C intakes to be 920 mg and 94 mg respectively. Moshfegh et al. (43) have reported usual intake levels of these nutrients as 892 mg and 91.8 mg both of which are within our estimated confidence intervals. While a national estimate of daily phytate intake in the US population is scanty, our analysis suggests a mean daily intake of 719 mg is plausible. We also estimated mean MFP intake to be 128 g/day which is consistent with the amount reported by Daniel et al. (44) based on NHANES 2003-2004 survey data.

Various studies have estimated iron absorption from the complete diet, particularly in the case of nonheme iron absorption. Among four studies that estimated nonheme iron absorption from typical US diets, the values ranged from 4.6 to 7.4 (7, 11, 16, 45). However where meal compositions were varied to increase or decrease intake of selected factors, reported nonheme iron absorption include values as low as 0.7 % and values higher than 10 % (6, 9, 10, 12). In this study, when estimated for different age and gender groups, nonheme iron absorption ranged from 1.9 % in men (19 to 50 year) to 7.3 % among children (3-8years). It was also clear that differences in nonheme absorption among the groups are largely due to differences in iron status between survey participants. Generally, groups that had high ferritin levels also had low nonheme iron absorption, and vice versa (Table 1). Our study also found that nonheme iron absorption was higher among women (all age groups combined) (5.55 %) than among men (2.56 %). This difference may be largely due to the lower serum ferritin concentration that was found among women compared to men. Women also had lower intakes of inhibitors of iron absorption such as calcium, nonheme iron and phytate than males. Tea equivalents intake did not differ

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significantly by gender and thus may not contribute to the discrepancy in iron absorption between the gender groups. With respect to race, iron absorption was lower among non-Hispanic Whites than among non-Hispanic Blacks or Mexican Americas. We can only explain these differences by disparities in ferritin concentrations and in the composition of the diet in the three race-ethnicity groups, since the algorithm that we used to estimate iron absorption does not include genetic and interpersonal attributes as explanatory variables. We found that non-Hispanic Whites had a higher ferritin concentration and tea intake, and a lower vitamin C intake than both non-Hispanic Blacks and Mexican Americans ($P<0.05$). Also, non-Hispanic Whites had higher intake of calcium and phytate and lower intakes of MFP than non-Hispanic Blacks ($P < 0.01$).

Using the two different approaches to estimate total iron absorption yielded similar values (15.49 % when nonheme absorption was adjusted to 15 µg/L ferritin, and 15.06 % when only nonanemic subjects with no iron stores were included). Our results therefore suggest that the bioavailabilty of iron in the US diet is 15 % rather than 18 % as is currently assumed. To validate our results, we considered data arising from three studies in which nonheme iron absorption was measured from the typical US diet consumed over a five-day period (7, 11, 45). From these studies we selected only those subjects with ferritin concentration of 15 µg/L or less. Total iron absorption for these subjects based on measured fractional nonheme iron absorption and a conventional heme iron absorption of 25 % was 15.5 % even though the number of subjects meeting this criteria was small (n=5). In their estimation of iron bioavailability from Western-type diets, Hallberg and Rossander-Hultén (13) obtained a value of 16.6 % for the United States diet. However, this estimate was based only on data for women. The researchers suggested that an average value for long term iron

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bioavailability in western type diets may be approximately 15 % among borderline iron deficient subjects, with a likely range of 14-17 %. In a systematic review of iron absorption from whole diets, Collings et al. (46) have suggested that even among individual with low iron stores, iron bioavailability may be less than 15 % particularly if they are consuming a low bioavailability diet. Their model suggests that individuals with ferritin concentrations of 12 and 15 µg/L consuming high bioavailability diets will absorb 13.9 and 11.8 % of nonheme iron respectively. This translates to 15.0 and 13.1 % total iron bioavailability respectively assuming that 90 % of the dietary iron is nonheme and that 25 % of heme iron is absorbed.

An important issue that deserves revisiting is the whole idea of estimating absorption using information from individuals with no iron stores (Ferritin ≤ 15 µg/L) or adjusting absorption to a ferritin level of 15 µg/L. This practice is questionable when we consider that the geometric mean of ferritin for the entire US population is well above this value. By basing estimation of absorption on individuals with no iron stores, we tend to obtain higher estimates for bioavailability than we would when considering the entire population. Further, this approach raises questions about iron absorption in individuals with high iron stores (5). On the plus side, the current methodology results in an upper bound of iron absorption (or maximum absorption) from the US diet, and thus is useful in setting lowest ceilings for the different DRIs for iron (13). When we estimated total iron absorption for all subjects without adjusting for serum ferritin by any of the two different approaches described earlier, the estimated mean absorption for the entire population was 5.8 %. This value is less useful, however because it hugely under-estimates iron absorption for individuals with low iron stores and at the same time overestimates absorption for individuals with very high iron stores. From a public health point of view, using the maximum

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absorption value as the national estimate is the lesser of two evils in that it leads to over-estimation of absorption, but only in individuals with adequate iron stores. For example if we estimate the prevalence of inadequate iron intake among men using the maximum iron absorption threshold, we are likely to under-estimate prevalence of inadequacy. However, it is also the case that we rarely find iron deficiencies among men.

Some limitations of this study are discussed below. First, since phytate intake was not part of the NHANES survey variables, we estimated it using the method described earlier. To minimize errors, we used phytate content values from a reliable source (37). Secondly, because it is not feasible from a practical point of view to measure iron absorption in a nationally representative sample of individuals, we estimated absorption using an algorithm proposed by Armah et al. (33). The algorithm we implemented is based on a complete diet and includes iron status as part of the model. As discussed in (33), the prediction model for iron absorption has been validated using published data with reliable predictive power. The algorithm, however does not capture the effect of genetic and interpersonal variability in iron absorption. Therefore, we predict mean iron absorption for all individuals with the same values of iron status and of the dietary factors included as explanatory variables in the model. Also, the algorithm was developed with data for adults 19 to 38 years old, and thus absorption prediction with this model may be most appropriate for this group. Polyphenols constitute one the main inhibitors of iron absorption yet consumption of polyphenols is not included in NHANES. To overcome this problem, we used tea, coffee and other polyphenol containing beverages intake expressed in black tea equivalents to approximate intake of polyphenols at the individual level. In

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conclusion, the results of this study suggest that the bioavailability of iron in the US diet is about 15 %, lower than 18 % assumed at present.

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Table 1 Summary of nutrients intake, nonheme iron absorption and serum ferritin concentration for subjects in NHANES 2001/2002[1,2]

	3-8 y (*n*= 836)	9-18y Boys (*n*=1107)	9-18y Girls (*n*=1113)	19 – 50y Men (*n*=1,127)	>50 Men (n=811)	19-50y Women (*n*=936)	>50y Women (*n*=701)	Total[3] (*n*= 6,631)
Vitamin C, *mg*	82 (74-90)	94 (86-103)	82 (72-92)	103 (95-111)	100 (91-108)	83 (76-90)	94 (87-101)	94 (89-99)
Calcium, *mg*	941 (897-984)	1177 (1084-1271)	868 (819-918)	1070 (1018-1123)	878 (827-929)	783 (728-837)	720 (687-754)	920 (890-950)
Tea[4], *cups*	0.10 (0.06-0.14)	0.12 (0.09-0.15)	0.14 (0.10-0.18)	0.35 (0.31-0.39)	0.43 (0.37-0.48)	0.30 (0.27-0.34)	0.45 (0.40-0.50)	0.30 (0.28-0.33)
MFP[5], *g*	77 (71-83)	127 (119-136)	88 (81-95)	182 (176-188)	141 (131-152)	113 (106-120)	88 (82-95)	128±2 (125-132)
Nonheme iron, *mg*	12.0 (11.3-12.7)	16.7 (15.9-17.5)	12.4 (11.8-12.9)	17.2 (16.3-18.0)	15.2 (14.2-16.3)	12.3 (11.8-12.8)	11.8 (11.2-12.3)	14.3 (13.8-14.7)
Phytate, *mg*	551 (516-585)	734 (696-773)	604 (571-637)	855 (809-900)	794 (717-872)	644 (584-704)	662 (585-738)	719 (684-753)
Absorption of nonheme iron[6], *%*	7.32 (6.85-7.83)	5.02 (4.82-5.23)	7.25 (6.92-7.60)	1.93 (1.84-2.02)	2.03 (1.89-2.18)	6.21 (5.96-6.46)	3.48 (3.26-3.72)	3.70 (3.61-3.80)
Serum ferritin[6], *µg/L*	24.9 (22.5-27.6)	36.4 (34.4-38.5)	24.7 (23.3-26.1)	131.7 (123.7-140.2)	131.0 (117.9-145.5)	29.5 (27.5-31.8)	69.7 (63.5-76.6)	58.4 (56.8-60.1)
HB[6], *g/dL*	13.1 (12.9-13.2)	14.6 (14.4-14.8)	13.6 (13.4-13.8)	15.6 (15.4-15.8)	15.0 (14.8-15.2)	13.5 (13.3-13.6)	13.8 (13.6-14.0)	14.3 (14.1-14.5)
CRP, *mg/L*	0.76 (0.65-0.87	0.77 (0.69-0.86)	0.87 (0.77-0.97)	1.60 (1.50-1.71)	1.98 (1.85-2.11)	1.90 (1.79-2.02)	2.40 (2.26-2.55)	1.61 (1.57-1.65)

[1]Estimates are for only subjects with complete data for estimating nonheme iron absorption
[2]Values are mean (95 % confidence interval)
[3]Excludes pregnant and lactating women
[4]1 cup (240mL) of black tea= 2 cups of iced tea or 1.5 cups of herbal tea or coffee
[5]MFP: Meat, fish and poultry
[6]Geometric mean

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Table 2 Percentiles of nutrients intake, nonheme iron absorption and serum ferritin concentration among subjects in NHANES 2001/2002[1]

Nutrient/Factor	10th	25th	50th	75th	90th	Mean
Vitamin C, *mg*	12 (11-13)	26 (24-28)	59 (55-64)	127 (120-133)	216 (207-227)	94 (89-99)
Calcium, *mg*	311 (294-324)	496 (465-522)	786 (759-812)	1180 (1149-1212)	1670 (1609-1719)	920 (890-950)
Tea[2], *cups*	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.07)	0.33 (0.28-0.42)	1.00 (1.00-1.00)	0.30 (0.28-0.33)
MFP[3], *g*	11 (5-17)	51 (46-56)	102 (99-106)	176 (170-182)	268 (259-278)	128 (125-132)
Nonheme iron, *mg*	6.1 (5.9-6.3)	8.7 (8.5-8.9)	12.3 (12.0-12.6)	17.5 (16.8-18.2)	24.6 (23.3-25.9)	14.3 (13.8-14.7)
Phytate, *mg*	240 (226-253)	365 (349-382)	567 (543-595)	881 (836-926)	1323 (1262-1415)	719 (684-753)
Absorption of nonheme iron[4], *%*	1.24 (1.18-1.29)	1.95 (1.87-2.01)	3.73 (3.59-3.85)	6.65 (6.43-6.86)	10.73 (10.33-11.06)	3.70 (3.61-3.80)
Serum ferritin[4], *µg/L*	15.0 (14.0-16.0)	27.0 (26.0-28.0)	58.0 (56.0-61.0)	135.0 (129.0-141.0)	241.0 (230.0-253.0)	58.4 (56.8-60.1)

[1]Estimates are for only subjects with complete data for estimating nonheme iron absorption (n=6,631); Values are percentile (95 % confidence interval)
[2]1 cup (240mL) of black tea= 2 cups of iced tea or 1.5 cups of herbal tea or coffee
[3]MFP: Meat, fish and poultry
[4]Values are geometric mean (95% confidence interval)

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Table 3 Dietary intakes and iron status by gender and ethnicity[1,2]

	Phytate, *mg*	MFP, *G*	Tea, *Cups*	Calcium, *mg*	Vitamin C, *mg*	Nonheme iron[3], *mg*	Ferritin[4], *µg/L*	Nonheme iron absorption[4], %
All (n=6631)	719 (684-753)	128 (125-132)	0.30 (0.28-0.33)	920 (890-950)	94 (89-99)	14.3 (13.8-14.7)	58.4 (56.8-60.1)	3.70 (3.61-3.80)
Gender								
Males (*n*=3456)	797 (756-838)	156 (151-160)	0.31 (0.27-0.35)	1041 (1001-1080)	102 (95-109)	16.3 (15.6-17.0)	92.2 (88.6-96.0)	2.56 (2.47-2.65)
Females (*n*= 3175)	633 (595-670)	98 (93-104)	0.29 (0.26-0.32)	788 (758-815)	85 (81-89)	12.1 (11.7-12.4)	35.5 (33.9-37.2)	5.55 (5.39-5.72)
Ethnicity								
Non-Hispanic White (*n*= 2870)	737 (691-784)	126 (122-131)	0.32 (0.29-0.36)	970 (929-1010)	89 (84-94)	14.5 (13.9-15.1)	61.0 (58.2-63.9)	3.50 (337-3.64)
Non-Hispanic Black (*n*= 1601)	617 (580-654)	150 (141-160)	0.21 (0.17-0.25)	723 (694-753)	106 (94-117)	13.7 (13.1-14.3)	53.8 (49.0-59.1)	4.39 (4.10-4.70)
Mexican American (*n*=1676)	693 (656-730)	131 (121-142)	0.19 (0.16-0.22)	910 (866-954)	108 (102-113)	14.2 (13.4-15.0)	46.8 (43.1-50.7)	4.49 (4.20-4.79)
Other Race - Including Multi-Racial (*n*= 193)	719 (604-834)	127 (108-145)	0.37 (0.26-0.48)	680 (602-757)	111 (93-128)	13.0 (12.2-13.9)	62.6 (51.7-75.8)	3.74 (3.23-4.33)
Other Hispanic (*n*=291)	708 (602-816)	109 (89-129)	0.31 (0.20-0.41)	853 (778-929)	102 (88-116)	13.7 (13.1-14.3)	52.9 (43.6-64.0)	4.07 (3.54-4.68)

[1]Estimates are for only subjects with complete data for estimating nonheme iron absorption (n=6,631); Values are percentile (95 % confidence interval)
[2]1 cup (240mL) of black tea= 2 cups of iced tea or 1.5 cups of herbal tea or coffee
[3]MFP: Meat, fish and poultry
[4]Values are geometric mean (95% confidence interval)

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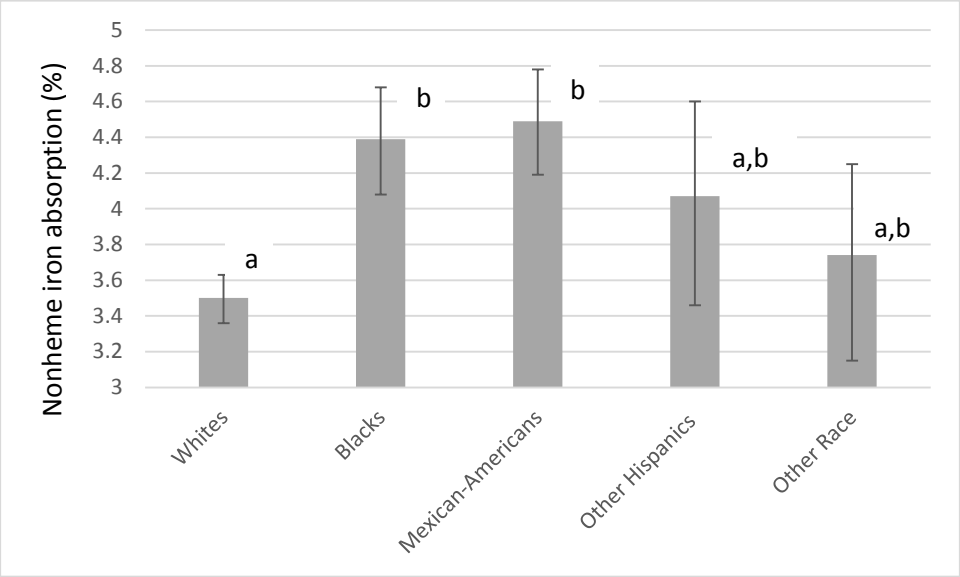

Figure 1A Nonheme iron absorption among different ethnic groups. Absorption values for bars with different alphabets are significantly different (*P < 0.005*).

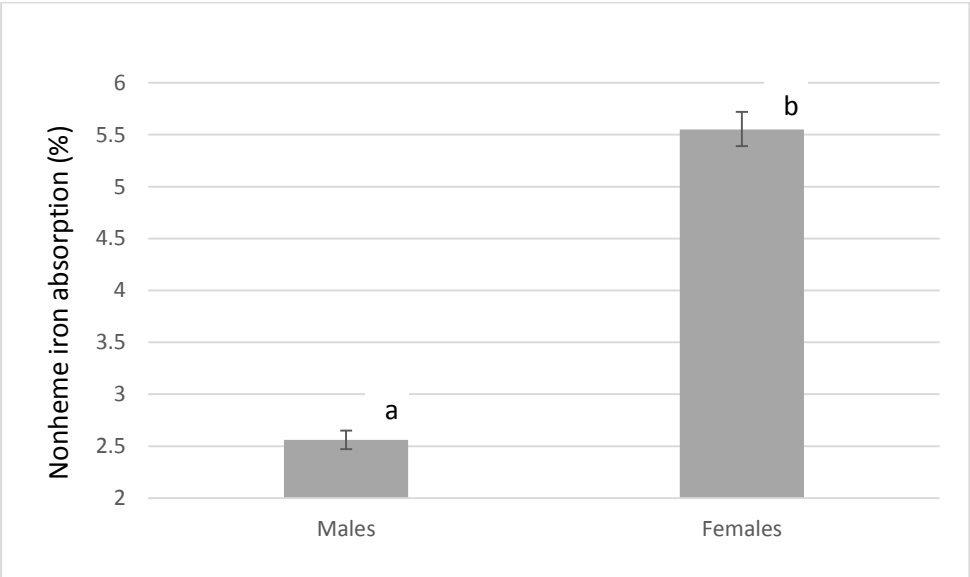

Figure 1B Nonheme iron absorption by gender. Different alphabets on bars indicate significant difference in absorption (*P < 0.0001*).

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CHAPTER 5. ADAPTATION OF IRON ABSORPTION IN HUMANS WITH HIGH PHYTATE DIET CONSUMPTION

A paper submitted to the *Journal of Nutrition*

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Abstract

Background: Phytate is one of the main inhibitors of nonheme iron absorption. High phytate consumption is of concern in developing countries because of the high prevalence of iron and zinc deficiency in these countries. **Objective:** In this study, we investigated whether individuals adapt to the inhibitory effect of phytate on iron bioavailability. **Design:** Female subjects with ferritin < 30 µg/L (n=28) were randomized into two groups of 14, after matching for ferritin. Each group consumed either high or low phytate foods that were provided for 8 weeks. Iron bioavailability from a high phytate test meal was measured using area under the curve (AUC) for serum iron at baseline and after the intervention. **Results:** Due to the dietary modification, phytate intake changed significantly (P <0.01) in both groups. No significant changes were observed in ferritin, transferrin receptor, hepcidin, and C-reactive protein concentrations in both groups. AUC increased significantly (P < 0.05) in the high phytate group (640 to 905 µmol/L) but the decrease (337 to 267 µmol/L) in the low phytate group was not

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[5] Corresponding author. Involved in study design, manuscript preparation and provided oversight of study.

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significant. We found a strong positive correlation between mean phytate intake and AUC (r = 0.70, P <0.0001) at post-intervention but not at baseline, and a negative correlation between hepcidin and AUC at both baseline (r = -0.43; P = 0.01) and post intervention (r = -0.41; P = 0.02). Phytate intake was also negatively correlated with hepcidin concentration at post-intervention (r = 0.40; P = 0.02). **Conclusion:** Our data suggests that individuals who consume a habitual high phytate diet may adapt to the inhibitory effect of phytate on iron absorption. Further studies are needed to explore the possible mechanism by which the adaptation occurs.

Introduction

Numerous studies have been conducted to investigate the effect of dietary factors on iron absorption (1-7). These dietary factors include meat, calcium, ascorbic acid, tea/polyphenols, phytic acid and nonheme iron. These, along with iron status of the individual, are well recognized determinants of iron absorption based on single meal absorption studies (8, 9). Studies have however suggested that the effect of dietary factors may be dampened in the usual/complete diet. In one study Cook et al. (10), demonstrated that the effect of these dietary factors on iron absorption is exaggerated in single meal studies. They reported that modifying the diet to contain high levels of meat and other enhancers resulted in only a small increase in absorption from the complete meal, while an exaggerated increase was observed in the single meals. Similarly an inhibitory diet, limited in meat and ascorbic acid and generous in phytic acid, calcium and polyphenols, consumed over two weeks showed a relatively lower reduction in iron absorption compared to what was observed in single meal. In other studies the effect of meat and ascorbic acid on nonheme iron absorption from a complete diet was shown to be

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marginal, compared to the reported enhanced effect (1, 4). In yet another study, no effect of 2 g ascorbic acid supplementation for 16 weeks or even 24 months on iron status was reported despite an increase in absorption from a single meal (11). Similarly, in a study designed to investigate the long-term impact of increasing ascorbic acid containing lime juice intake on iron status among Mexican women who were iron deficient, no improvement in iron status was found in the experimental group compared to the control group and both groups remained iron deficient at the end of the eight month intervention (12). Our recent study also showed no significant effect on iron status in premenopausal women who consumed soybean diet for 10 weeks (13).

The dampened effect of dietary factors may be due to the interaction of other meal components. Another possible explanation for the diminished or lack of effect of dietary factors in long term studies is that individuals may adapt to the effect of these factors. Various studies, including both human and animal research, have investigated this hypothesis (14-16). In a study with male subjects after matching for ferritin, nonheme iron absorption decreased among subjects consuming high bioavailability diets, and increased among those consuming low bioavailability diets (15). The high bioavailability diets included close to 400 g/day of meat or poultry, high vitamin C, and no coffee or tea. The low bioavailability diet included tea and plenty of whole grain cereals and legumes, limited amounts of poultry and fish, with no meat. On the contrary, Brune et al. (14) have reported that no intestinal adaptation to a high phytate diet occurs based on a study in which iron absorption was measured from wheat and bran rolls among vegetarians and non-vegetarians. In this study, we investigated whether individuals adapt to the inhibitory effect of phytate on iron absorption after an eight week modification in phytate intake

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using the area under the curve (AUC) for serum iron method. The AUC method was validated by Conway et al. (17) by showing a strong positive correlation with erythrocyte incorporation of isotopic iron. We hypothesize that fractional nonheme iron absorption from a high phytate test meal will increase in subjects consuming a high phytate diet and decrease or not change among those consuming a low phytate diet for eight weeks.

Methods

Subjects. Female subject were recruited for this study by sending mass e-mail to all female students in Iowa State University in the Spring of 2013. A total of 113 students responded, however only 97 participated for initial screening **(Figure 1)**. Prior to screening, they were required to read and sign an informed consent document, and all procedures, potential risks and benefits were explained to them. The study protocol was approved by the Institutional Review Board at Iowa State University (IRB# 12-470). Potential subjects were required to go through three different stages of screening. At first screening they completed health and medical history questionnaire and their height and weight were measured for BMI assessment. Subjects who were eligible came back for ferritin eligibility ($\leq$30 ug/L) and then for pregnancy test. We determined that 14 subjects were needed in each group to determine a change in AUC by one-third (33 %) as significant with 80 % power and at an alpha level of 0.05 (15, 17). A total of 32 subjects were recruited for the study based on ferritin ($\leq$30 ug/L) and BMI within the normal range (18.5-24.9 kg/m^2). Participation criteria included non-smoking, non-lactating, non-pregnant, not taking any drug that interferes with iron absorption, and should not have any gastro-intestinal disease/condition that can affect absorption. The 32 recruited subjects were first matched for ferritin and then randomized, into either high or low

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phytate diet group (n=16 per group). The randomization and allocation of subjects was done by a person who was not involved in the study using computer based randomization.

Study protocol. After recruitment, subjects were asked to keep a three day dietary record (two weekdays and a weekend) prior to reporting to the Nutrition and Wellness Research Center (NWRC) at Iowa State University for baseline absorption measurement. Iron absorption from a high phytate test meal to which 10 mg iron as $FeSO_4$ has been added was measured using the AUC for serum iron (17). The test meal was composed of a small corn tortilla (27.2g), 100g black beans (drained from can), 50g cooked white rice, 30g salsa and 120g orange juice. Subjects were asked to fast overnight before coming to the NWRC for the first absorption measurement. A baseline blood sample was collected after which test meal was administered. After consuming the test meal, blood samples were collected every 30 minutes for 4 hours. After the first absorption measurement, subjects participated in an eight week dietary intervention in which each subject consumed either high or low phytate diets. All food needed for the dietary modification was supplied to subjects. Their first two-week food supply was given on the day they came for the baseline multiple blood draw. Subjects reported to the NWRC every two weeks to pick up food. The high phytate group received whole grain ready-to-eat cereals, whole wheat pasta/spaghetti, tortillas, bagels, bread and dinner rolls, corn tortillas, brown rice, canned black beans, edamame and tofu, and were encouraged to consume generous amounts of nuts and other legume products high in phytate. The low phytate group received similar foods made from refined wheat and white rice, eggs and cheese, and were instructed to avoid high phytate foods. At weeks 4 (data not shown) and 7, the

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subjects were asked again to keep three day dietary records. Post intervention absorption measurement with the same meal that was given at baseline was done after the eight week dietary intervention. Four subjects dropped out during the study (One got an internship and could not continue with the study, one got sick but not related to the treatment, one subject did not want to follow the dietary modification and opted to quit, and the last one had difficulty with the multiple blood draws and decided to quit.), leaving 28 who completed the study (14 in each group). To assess compliance, subjects were required to write down daily all the foods they consumed from what they were provided. They were required to incorporate the foods they had been provided in at least two of their daily meals. Also at the end of the study, they were asked to provide information on how frequently they consumed specified high phytate foods listed in a food frequency questionnaire. Compliance was estimated as the percentage of days in which they included the foods they were provided in at least two of their daily meals. Also from the food frequency questionnaire, the total number of times they have consumed high phytate foods over the eight week intervention period was estimated as a measure of compliance.

Blood sample analysis. Before administering the test meal, two separate fasting blood samples were collected, one for measurement of hepcidin, and the other for measurement of initial (T_0) serum iron, C-reactive protein (CRP), transferrin receptor and serum ferritin. Multiple blood samples collected after the test meal was consumed were analysed for serum iron by the certified lab (Quest Diagostics, Inc), and results were used to construct serum iron curve for each subject during a four hour period. Blood samples collected were centrifuged and serum was aliquoted into microcentrifuge tubes and

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frozen until they were analysed. Aprotinin (Fisher Scientific, Pittsburgh, PA) was added to the hepcidin sample and it was frozen at -80ºC. Samples for measurement of CRP, transferrin receptor and ferritin were stored at -20ºC until the end of the study when all samples were analysed. CRP, Hepcidin (Hepcidin-25), ferritin and transferrin receptor were measured using Enzyme Linked Immunosorbent Assay (ELISA). Kits for the measurements were obtained from American Laboratory Products Company (ALPCO Diagnostics, Salem, NH) for CRP, Penninsula Laboratories (San Carlos, CA) for hepcidin-25, and Ramco Laboratories Inc. (Stafford, TX) for ferritin and transferrin receptor. Dietary data were analysed using the Nutrition Data System for Research (NDSR, University of Minnesota, MN).

Statistical analysis. Data were analysed using the Graphpad Prism 6 (GraphPad Software, Inc. La Jolla, CA). For all variables, analyses were done for all 28 subjects who completed the study (14 per group). AUC for serum iron as a primary outcome was estimated for each subject and the geometric mean was reported for each group. We also estimated percentage iron recovery at baseline using the method described by Conway et al. (17) to correlate with AUC. We estimated total body iron using the equation by Cook et al. (18). For iron status indices and AUC, the data were log transformed for statistical analysis and the original values were recovered by back-transforming. Geometric means were reported for the iron status biomarkers except for body iron where median was reported because some of the values were negative. Median, 10th and 90th percentiles were reported for the dietary variables. Paired t-test was used to compare baseline and post values of biomarkers of iron status, dietary intakes and AUC within each group. Comparisons between groups of baseline or post measurements were done using

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independent t-test. Differences were considered statistically significant at $P \leq 0.05$. Pearson correlation was used to assess relationships among AUC, phytate intake and hepcidin concentration. For correlation analyses data for all 28 subjects were combined instead of analysing by groups.

Results

Of the 28 subjects who completed the study, there were 22 Whites, 3 Asians, 2 Blacks and 1 Latino. The mean (range) height, weight, and BMI of all 28 subjects at baseline were 167 cm (156-180 cm), 62.1 kg (49.0-75.5 kg), and 22.3 kg/m^2 (18.5-24.6 kg/m^2). Geometric means for serum ferritin, transferrin receptor, CRP and hepcidin concentrations were 19.0 µg/L, 5.2 µg/mL, 0.77 mg/L and 2.3 ng/mL. None of the above mentioned measurements differed significantly between the high and low phytate groups at baseline, with the exception of hepcidin concentration which was higher in the low phytate group than the high phytate group ($P = 0.019$). **Table 1** compares the baseline and post-intervention dietary intakes of subjects by groups. There were no significant changes in nutrients intake in both groups apart from phytate. Compliance as estimated by the percentage of days in which subjects incorporated provided foods in their daily meals was 87 % in the low phytate group and 96 % in the high phytate group. Over the 8 week intervention period, the high phytate group incorporated high phytate foods 271 times on average compared to 27 in the low phytate group. Based on the changes in dietary pattern, phytate intake increased significantly ($P < 0.01$) among the high phytate group and decreased significantly ($P < 0.001$) among the low phytate group.

Changes in biomarkers of iron status and measures of iron absorption are shown in **Table 2**. Ferritin, transferrin receptor, body iron, hepcidin, and CRP did not change

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significantly within groups. **Figure 2,** shows the serum iron curves for the high and low phytate groups at baseline and post intervention. The curves shown are based on average values for each group. For each individual, the curve was obtained by calculating the change in serum iron from baseline at 30 minutes intervals for 4 hours after consuming a high phytate test meal. AUC increased significantly (P<0.05) by 48 % in the high phytate group from 640 to 905 µmol/L, whereas the 21 % decrease in the low phytate group (337 to 267 µmol/L) was not statistically significant (Table 2). When we estimated percentage iron recovery (at maximum) as an estimate of absorption based on the calculation by Conway et al. (17), the correlation with AUC was 0.86 (P <0.0001) at baseline. We did not include an estimate at post intervention because we did not have all the measurements needed for the calculation. In the low phytate group, the geometric mean (range) of absorption estimated using this method was 3.1 % (0.4-7.2 %), while it was 5.5 % (1.0-9.3 %) in the high phytate group.

The correlations among phytate intake, AUC and hepcidin (for all subjects, n=28) are shown in **Figure 3**. At baseline, no significant correlation was observed between phytate intake and AUC (Figure 3 A). However after the intervention (Figure 3B), AUC was significantly correlated with phytate intake (r = 0.70; P < 0.0001). Hepcidin concentration was inversely correlated with AUC both at baseline (r = -0.43; P = 0.01) and at the post-intervention (r = -0.41; P = 0.02) (Figure 3 C and D, respectively). There was no significant correlation between hepcidin and phyate intake at baseline (Figure 3E). However, at post-intervention, as phyate intake increased hepcidin concentration decreased (r = -0.40, P = 0.02) (Figure 3F).

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Discussion

Phytate is known as one of the major inhibitors of iron absorption (19). Higher inositol phosphates like inositol hexaphosphate (IP_6) and inositol pentaphosphate (IP_5) in particular are known to bind to iron and make it unavailable for absorption (20). Hallberg et al. (21) have reported a dose dependent inhibition of sodium phytate on iron absorption from meals consumed together with radiolabeled wheat rolls. A key reason for the concerns about phytate is that it is a major component of the staples in many developing countries where iron deficiency prevalence is high (22). Interestingly, studies that have investigated the effect of dietary factors on iron absorption in complete diets have suggested that their effect may be dampened when the whole diet is considered (10). Particularly for phytate, no study has investigated its effect on iron absorption from the whole diet assessed over a short or long term to the best of our knowledge. In order to determine whether individuals adapt to the effect of phytic acid on iron absorption, Brune et al. (14) compared the effect of bran on iron absorption between a vegetarian group and a nonvegetarian control group, and found no significant difference, suggesting no adaptation. However the average daily phytate intake among the vegetarian group was 323 mg/day, which is well below what has been reported for many countries. For example, median phytate intake in the United Kingdom was estimated to be 809 mg/day (23). In most developing countries, daily phytate intake values are even higher (24). In this study we investigated whether individuals adapt to the inhibitory effects of phytate on iron absorption after long-term phytate consumption. Baseline phytate intakes were 715 and 793 mg/day for low and high phytate groups respectively which are comparable with reported average intake of 750 mg/day for US adults (25).

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The results of this study also indicate no effect of the intervention on the biomarkers of iron status. In the high phytate group there was an increase in serum ferritin and body iron and a decrease in transferrin receptor while the low phytate group showed a decrease in ferritin concentration. However, none of these changes were significant which was expected considering short duration of dietary modification in our study. These results are similar to the observation by Hunt and Roughead (15), who found that a ten week dietary intervention with a high or low bioavailability diet did not affect blood indices of iron status. Since hepcidin concentration was higher in the low phytate group than the high phytate group and based on its inverse relationship with iron absorption, it was not surprising see to see that baseline absorption and AUC was lower in the low phytate group compared to high phytate group. Baseline AUC among the high phytate group was about twice that among the low phytate group (640 vs 337 µmol/L). Even when we estimated absorption at baseline using the maximum iron recovery method, the difference was similar (5.5 % vs 3.1 %).

Hepcidin is known as a key regulator of iron absorption. It regulates iron absorption through ferroportin, the main iron exporter from intestinal cells and macrophages. Hepcidin binds to ferroportin and prevents the export of iron, resulting in the internalization of the ferroportin and subsequent reduction in iron absorption. Thus higher hepcidin concentration leads to lower iron absorption and vice versa (26-29). Our results of hepcidin showing negative correlation with AUC at both baseline and post-intervention support the above relationship. This strong correlation also suggests that the AUC for serum iron is a good measure of iron absorption despite the fact that it may not be as sensitive as isotopic methods. The AUC method was validated by Conway et al.

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(17) by showing a strong positive correlation with erythrocyte incorporation of isotopic iron.

There was a strong positive correlation between phytate intake and absorption at post intervention ($r = 0.70$; $P < 0.0001$), supporting the assertion that long-term phytate intake may dampen the inhibitory effect of phytate on iron absorption. In a similar study by Hunt and Roughead (15) nonheme iron absorption increased among subjects consuming a low bioavailability diet and decreased among those consuming a higher bioavailability diet, implying that people adapt to the effect of dietary factors that affect iron absorption. At baseline, phytate intake did not show any correlation with absorption. This was probably because of the distribution of phytate intake. At baseline, the 10th and 90th percentiles for phytate intake were 368 mg and 1067 mg respectively, which is a smaller range compared to post intervention, when the 10th and 90th percentiles were 318 and 1521 mg respectively. For the same reason, there was no significant correlation between baseline phytate intake and hepcidin concentration, even though a significant negative correlation was found between the two variables after the dietary modification. While the results of our study suggest that individuals may adapt to the inhibitory effect of phytate on iron absorption, this observation was not explained by any of the measured biomarkers of iron status since none of them changed significantly after the eight week dietary modification. However, based on the correlations found among hepcidin, AUC and phytate, we cannot rule out a role of hepcidin in the adaptation in iron absorption. Future studies are needed to further investigate the mechanism. The authors would like to acknowledge the following limitations of this study. First, compliance of the dietary intervention was based solely on records kept by subject since it was not control feeding

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study. Secondly, subjects in both groups were not required to consume specific amounts of the high or low phytate foods they were provided. This was in part to create a condition similar to real life scenario and to accommodate individual differences in food consumption.

In conclusion, the results of this study suggest that among individuals with habitual high phytate diet consumption, the inhibitory effect of phytate on iron absorption may be dampened implying an adaptation to the inhibitory effect of phytate. The findings of this study also imply that dietary staples in developing countries can be biofortified with iron to reduce iron deficiency despite their high phytate contents since habitual consumption of these foods may result in adaptation to the inhibitory effect of phytate.

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Table 1 Nutrients intake[1] at baseline and post intervention among high and low phytate groups

	High Phytate (*n*=14)		Low phytate (*n*=14)	
	Baseline	Final	Baseline	Final
Fat, *g/1000kcals*	37 (30, 40)	37(30, 43)	38 (27, 44)	33 (23, 41)
Protein, *g/1000kcals*	36 (26, 48)	36 (29,50)	38 (31, 53)	38 (33, 46)
Vitamin C, *mg*	91 (41, 207)	85 (28, 192)	74 (20, 168)	64 (30, 117)
Calcium, *mg*	765 (540, 1278)	805 (571, 1152)	709 (386, 1294)	818 (583, 1362)
Iron, *mg*	13.4 (8.1, 20.8)	13.7 (9.7, 21.0)	12.9 (9.7, 19.4)	14.4 (11.2, 16.9)
Phytate, *mg*	793 (396, 1094)[a]	1122 (916, 1602)[b]	715(362, 949)[c]	357(300, 487)[d]

[1]Values are median (10th, 90th percentiles). Values with different superscripts are significantly different ($P \leq 0.05$) within the same group based on paired t-test.

Table 2 Iron status biomarkers and serum iron[1] at baseline and post intervention

	High Phytate (*n*=14)		Low Phytate (*n*=14)	
	Baseline	Final	Baseline	Final
Serum ferritin, *µg/L*	17.4 (15.9, 19.1)	18.7 (17.0, 20.6)	20.7 (17.3, 24.8)	18.6 (16.1, 21.5)
Transferrin receptor, *µg/mL*	5.0 (4.5, 5.6)	4.7 (4.2, 5.3)	5.5 (4.6, 6.5)	5.5 (5.1, 5.9)
Body iron, *mg/kg body weight*	2.8 (0.6, 5.7)	3.4 (0.6, 6.1)	3.3 (-1.5, 7.9)	3.2 (-0.4, 5.9)
Hepcidin, *ng/mL*	1.5 (1.1, 2.0)	1.6 (1.2, 2.2)	3.6 (2.8, 4.7)	3.8 (3.2, 4.5)
C-reactive protein, *mg/L*	0.53 (0.40, 0.69)	0.67 (0.50, 0.90)	1.13 (0.70, 1.60)	1.46 (1.09, 1.96)
AUC for serum iron, *µmol/L*	640 (537, 764)[a]	905 (765, 1070)[b]	337 (269, 421)	267 (213, 334)
Maximum iron recovery, *%*[2]	5.5 (4.9-6.1)	NA	3.1 (2.4-3.9)	NA

[1] Values are geometric mean (± SE), except for body iron where median (10th, 90th) were reported because there were negative values and geometric mean could not be computed. Within the same group, values with different superscript are significantly different ($P < 0.05$) based on paired t-test.
[2] Not available at post intervention because hemoglobin concentration that was needed for calculations (17) was not measured.

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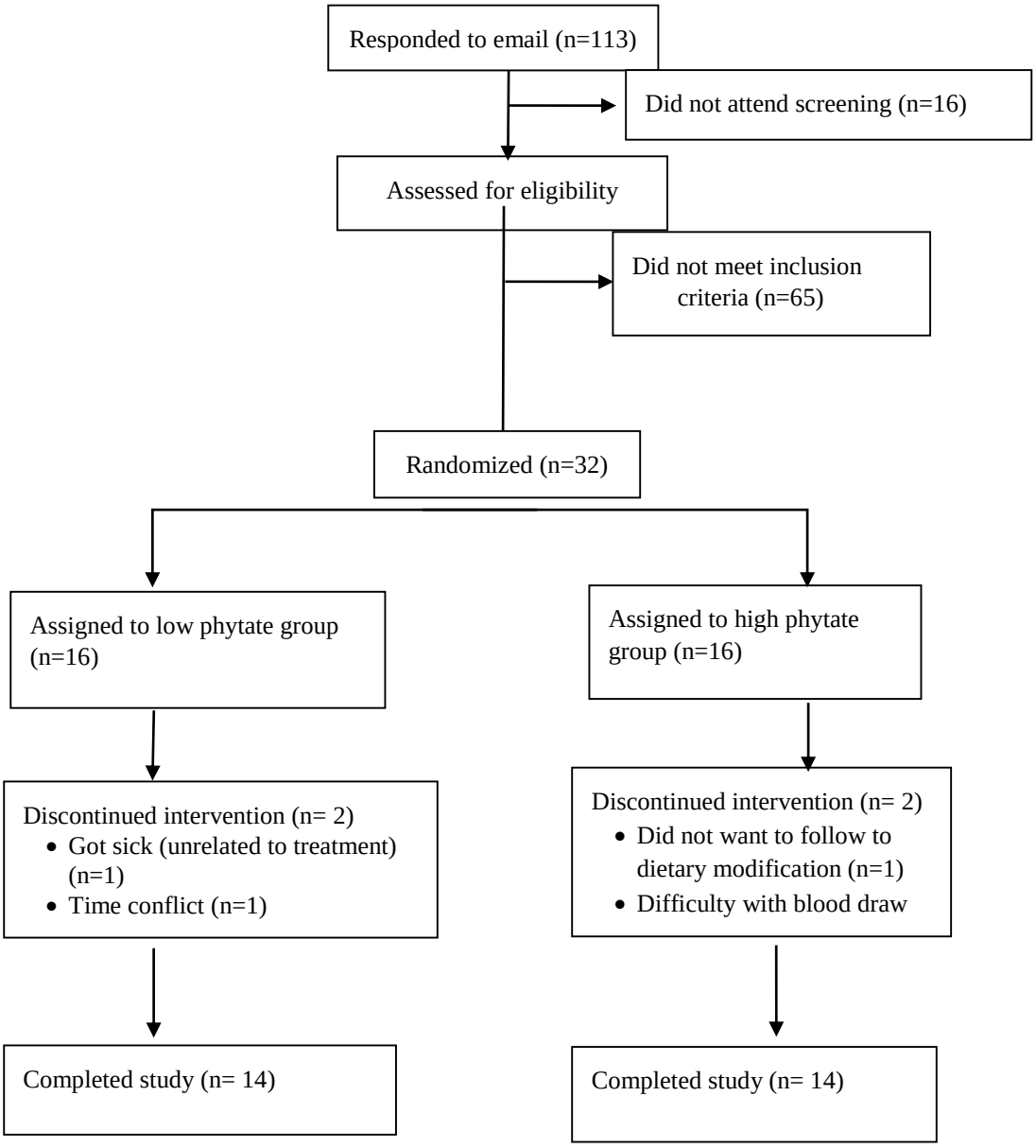

Figure 1 Subject selection

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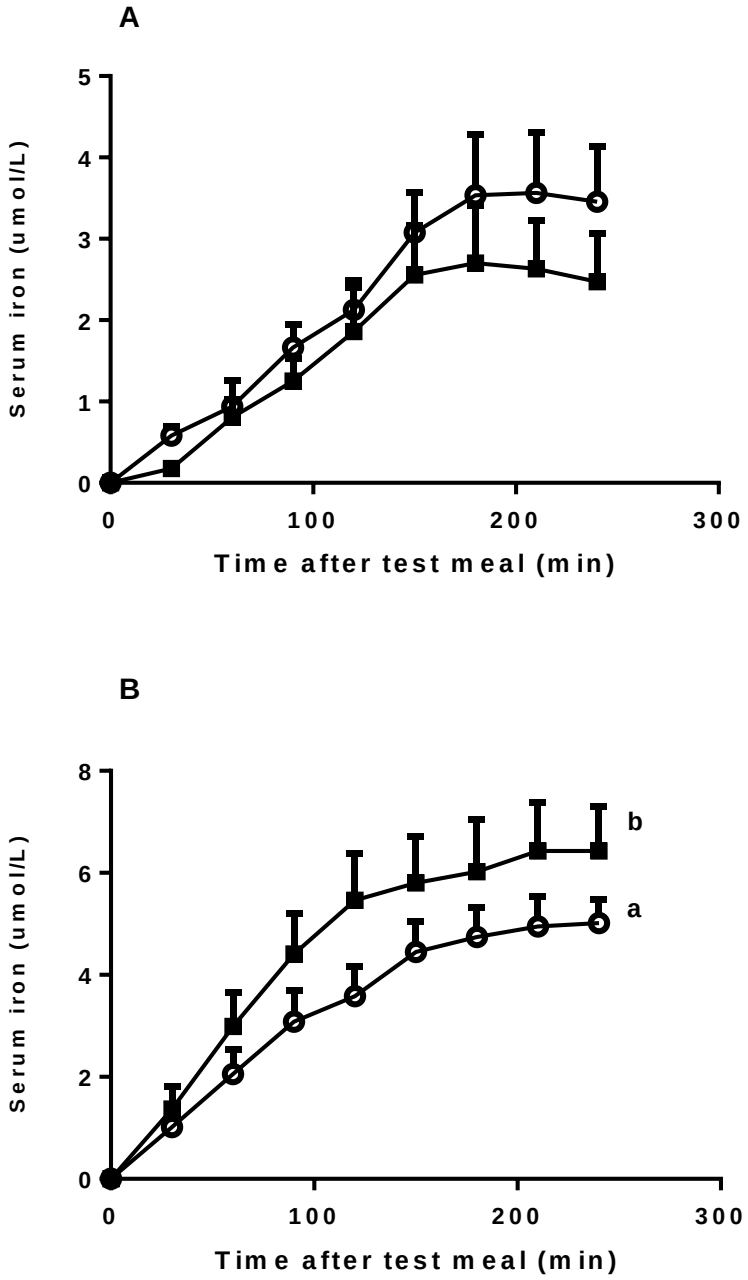

Figure 2 Serum iron curve at baseline (**o**) and post intervention (■) for low (n=14) and high (n=14) phytate groups. Values are mean + SE. Panel A and B represent serum iron curves for low phytate and high phytate groups. The AUC for serum iron curves with different superscripts were significantly different ($P \leq 0.05$) based on paired t-test.

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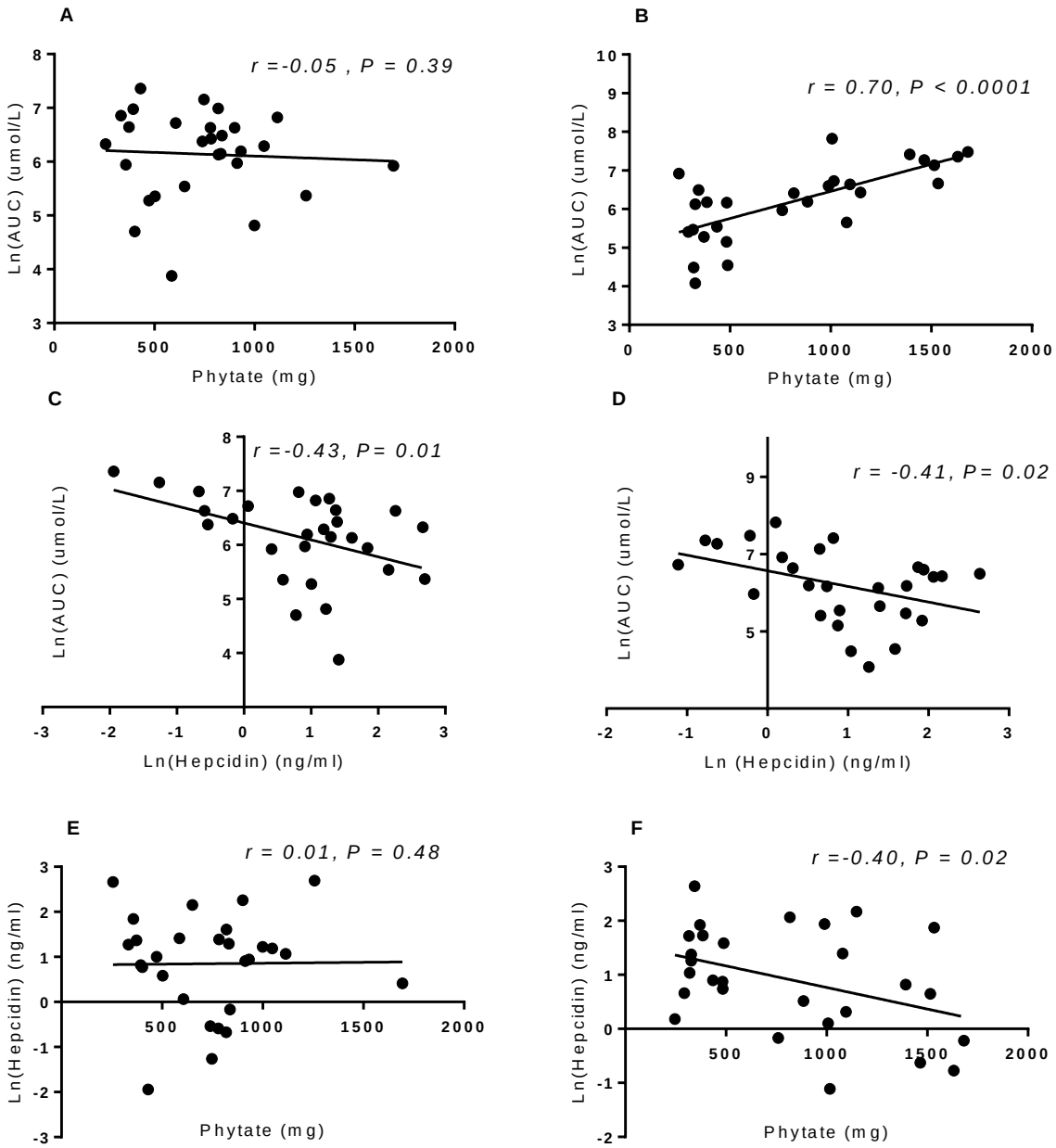

Figure 3 Correlations between phytate intake and AUC at baseline (A) and post intervention (B); between hepcidin concentration and AUC at baseline (C) and post intervention (D); between phytate intake and hepcidin concentration at baseline (E) and post intervention (F). Values are based on Pearson correlation test (n=28).

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CHAPTER 6. GENERAL CONCLUSIONS

In this study, we have investigated iron bioavailability using statistical and human models. We have developed a new algorithm that can be used to reliably estimate nonheme iron absorption from complete diets. The findings of this study affirm the fact that iron status is a more prominent predictor of nonheme iron absorption than dietary factors. Among the dietary factors, only phytic acid was marginally significant in explaining variability in nonheme iron absorption. Interperson variability also contributed significantly to the difference in nonheme iron absorption. Since it is not obvious what this individual difference may be, future studies are recommended to further investigate these interperson differences. The second study also shows that the percentage iron bioavailability from the US diet is 15 %, which is less than the value of 18 % used in developing the DRIs for iron. This finding is very important in reassessing the current DRIs for iron. It is also relevant in other aspects of iron nutrition such as program planning and dietary guidance. In the third study, we found that regular consumption of high phytate food may dampen the inhibitory effect of phytate on iron absorption. This study has implications on the biofortification of high phytate staples in developing countries. Since individuals in these countries consume high phytate foods habitually, high phytate staples can still be biofortified with iron to improve iron nutritional status in these countries. A major gap in this research is identifying the mechanism underlying this observation. While hepcidin was considered a possible mediator of the effect of long term phytate consumption, our data did not support this hypothesis, neither did any of the iron status biomarkers explain the mechanism. Future studies are therefore recommended to investigate the mechanism of adaptation in iron absorption.

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